



**Cloning and Expression of Antimicrobial Peptides
from *Vigna subterranea* (Bambara Groundnut)**

By

Saidat Olajumoke Rabi

212018132

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Master of Environmental Health

Faculty of Applied Sciences

Cape Peninsula University of Technology

Supervisor: Dr Richard Mundembe

Co-supervisor: Dr Vanessa Jackson

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ABSTRACT

Antimicrobial Peptides (AMPs) are short peptides of about 45 - 54 amino acids that exhibit antibacterial and antifungal activities. Plant defensin is a type of AMP in plants which belong to a family of cationic peptides with a characteristic 3D folding pattern held in place by four disulfide bridges. AMPs especially defensins have been identified to have a huge biotechnological potential and are being patented for many applications.

The aim of this work was to clone an antimicrobial peptide from *Vigna subterranea* and characterise it with bioinformatics analysis. 4 sets of primers were synthesized according to the sequences of conserved regions in AMPs i.e. defensins from legumes like *Vigna unguiculata*, *Vigna radiata*, *Cicer arietinum* and *Cajanus cajan*, amongst others, which have defensins with only a few sequence differences. The primers were designated VsDef P1 to P4. Using *Vigna subterranea* total genomic DNA as a template, fragments of expected sizes were successfully amplified and cloned into the pDRIVE vector and used to transform *Escherichia coli* JM109 cells in each case. Representative clones were sequenced and analysed using BLAST from National Center for Biotechnology Information. However, only the VIG clone was shown to be a *bona fide* defensin (over 90% identity, E-value of 1×10^2 , 99% query coverage of the nucleotide sequence, compared to *Vigna unguiculata* defensin). Based on this high sequence identity, a new pair of primers VsDef P5 was designed based on the *Vigna unguiculata* defensin sequence to specifically amplify the complete *Vigna subterranea* defensin gene, hereafter called *VsDef1*. Attempts to clone *VsDef1* were however unsuccessful, and evidence of clone deletion and insert re-arrangement of insert DNA was observed.

Direct sequencing of the PCR product demonstrated that it was indeed the complete *VsDef1* pre-protein, composed of 433 nucleotides. In silico translation and analysis showed that *VsDef1* has an intron at position 105 – 259 of the nucleotide sequences and encodes for a 78 amino acid peptide. Phylogenetic analysis revealed to be similar to the sequence of the defensins for *Vigna unguiculata* (96%), *Vigna radiata* (95%), *Vigna angularis* (95%) and *Phaseolus vulgaris* (93%) on the NCBI database. The three - dimensional structure of the peptide was modelled with SWISS-MODEL expasy and the structure was found to include one α - and three β domains, similar to those of other defensins.

The failure to identify *VsDef1* clone in a *V. subterranea* library and the failure to recover its cDNA clone are consistent with the hypothesised toxicity of *VsDef1* to *Escherichia coli*. It is

suggested that a different host, such as yeast, should be used in the future. The *VsDef1* mRNA levels in germinating *V. subterranea* seeds was however successfully investigated using real-time reverse transcription quantitative PCR. *VsDef1* mRNA is present in both the testa and embryo of dry seed and will persist through the early stages of seedling growth. This demonstrates the importance of *VsDef1* in fighting off infection during germination in order to ensure successful germination.

It is therefore essential to characterise more antimicrobial peptides from *V. subterranea*. The diversity of AMPs and their patterns of expressed genes will enable understanding of complex regulatory networks, which will likely enable identifying of genes involved in diseases and new biological processes.

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DEDICATION

I dedicate this to my mother and father

RESEARCH OUTPUTS

The research outputs below represent the contribution of the candidate to development and knowledge in science:

- Publication (submitted)

Rabiu, S.O., Jackson, V. & Mundembe, R. In press. Sequence characterization of *VsDef1*, a novel antimicrobial peptide isolated from *Vigna subterranea*. Submitted to *Biotechnology Reports*.

- **Presentation**

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ACRONYMS

- °C: Degrees Celsius
- µg: microgram
- aa: Amino acid(s)
- AMP: Antimicrobial peptide
- Amplicon: PCR products
- BLAST: Basic Local Alignment Search Tool
- bp: Base pair(s)
- cDNA: Complementary DNA
- DNA: Deoxyribonucleic acid
- kDa: Kilo Dalton(s)
- M: Molar
- mL: millilitres
- mM: Milli molar
- m: meters
- mm: milli meters
- MW: molecular weight
- NCBI: National Centre for Biotechnology Information
- PCR: Polymerase Chain Reaction
- RNA: Ribonucleic acid
- RT - PCR: Reverse Transcriptase Polymerase Chain Reaction
- Real Time RT–qPCR: Real-Time Reverse Transcriptase Polymerase Chain Reaction

CHAPTER ONE

INTRODUCTION

1.1. General Overview

Vigna subterranea (Bambara groundnut) is an orphan crop that originated from Africa but it is also grown in Asia and other continents (Hillocks *et al.*, 2011). The common name 'Bambara' seems to be derived from the Bambara tribes that are found in upper Niger close to Timbucto. As early as the 1600s, the plant was known in Brazil. Slavers, Portuguese voyagers carried it across the Atlantic, possibly via germplasm swaps which introduced three main foods - cassava, peanut and maize to Africa. Marcgrav de Liebstad (1648) recorded the crop as a native crop in Brazil. Du Petit-Thouars (1806) came across the crop in Madagascar under the name *voanjo* after the crop has gone missing in literature for over 158 years. Afterwards, the crop was only confirmed as a native African crop after ten years (National Research Council NRC, 2006).

In the early seventeenth century, *V. subterranea* became known in Surinam and Brazil, then it spread out to Indonesia and Philippines. In Africa, it is the third most cultivated grain legume crops in lowland tropics area after groundnut and cowpea. However, renewed interest for its cultivation has increased in recent years in the arid savannah zones including Madagascar (Department of Agriculture, Forestry and Fisheries DAFF, 2016). Though the first place where it was cultivated was not known but it is thought to have originated in the northern Cameroon and northeastern Nigeria, though wild types are still available in tropical eastern Africa (National Research Council, 2006).

It is a legume which provides a balanced diet due to its balanced proportions of carbohydrates, vitamins and proteins, amongst others. It is an essential part of food crops cultivated by poor farmers but are not rated as important by corporate agriculture because they are not economically important in the market. The resource-poor communal farmers like the crop and have maintained the germplasm for hundreds of years.

In South Africa, *V. subterranea* is only grown for subsistence reasons by local people though it is now being sold as a boiled snack in local markets. The major cultivation areas in South African provinces include Kwazulu-Natal, Mpumalanga, Northwest, Limpopo and Gauteng. It is often by itself or intercropped with meons, cowpea and maize. Less to no

information is available about agronomic practices for this crop because it was neglected by National Research Institutes in the past (DAFF, 2016).

With no reliable figures for production volume, it is estimated that West Africa produced 330 000 tons in the early 1980s. The main producers were Nigeria, Burkina Faso, Mali, Cameroun, Niger, Ghana and Ivory Coast (Plant Resources of Tropical Africa, 2015). There are seven varieties of *Vigna subterranea* seeds: black ones with small kernels that mature early, red ones with large kernels that mature late, the ones with large kernel and cream or black eye produce good yields, cream or brown eye ones with a medium-size kernel and good yield, cream or no eye ones with tiny kernels and pods though low yield, speckled/spotted ones with purple colour but kernels are small, brown ones which varies continually between light and dark brown (DAFF, 2011).

Although, it is like other legumes but contains a high proportion of amino acids with sulphur. Most of its genotypes have higher methionine and cysteine content than other legumes (National Research Council, 2006). *Vigna subterranea* can adapt to adverse environmental conditions such as poor soils and drought, making it thrive better than other legumes, such as cowpea and groundnuts in arid conditions. Antimicrobial peptides (AMPs) are thought to be one of the mechanisms used by *V. subterranea* to fend off infections during critical stages of germination.

Somta *et al.* (2011) analysed its genetic diversity using Simple Sequence Repeat (SSR) markers which allows assessment of how diverse the alleles and Polymorphism Information Content (PIC) value are in *V. subterranea* in contrast to the plants that falls in the *Vigna* genus. They found that *V. subterranea* from West Africa - Mali, Senegal and Burkina Faso had the most diverse genes excluding those from Cameroon or Nigeria. The average diversity of genes in *V. subterranea* is 0.55 which is slightly higher than the one of mungbean (0.41) but it is less than that of azuki bean (0.74).

The seeds of this legume are used to make various meals which form crucial part of diet and used as gift exchanges and in rites. It is grown mainly for its seeds, which are prepared by boiling and consumed as a pulse. Often, maize or plantain is added to the seeds before boiling. Porridge is made from roasted seeds ground into flour. They are also added to maize flour to enrich traditional preparations. Occasionally soaked seeds are ground into a paste which is fried or steamed to make dishes. In the cultivation season during the 'hungry gap' - when immature crops cannot be harvested and stores are bare, immature seeds are often eaten as a snack when boiled with salt. The leafy shoots and seeds are fed to poultry

and pigs. The leaves are used to treat infected wounds and inflammation, sap of the leaves is used to treat epilepsy when applied to the eyes, and the roots are known to be as an aphrodisiac in Senegal. The seeds are used to make fermented meals comparable to *Glycine max* tempeh as well as vegetable milk. In Nigeria, the Igbos treat sexually transmitted diseases with the plant (Plant Resources of Tropical Africa, 2015).

1.2. Significance of antimicrobial peptides (AMPs)

Since 1939, antimicrobial peptides (AMPs) termed host defense peptides have been classified among life classes ranging from prokaryotic organisms to human beings (Nawrot *et al.*, 2014). Plant antimicrobial peptides (AMPs) are proteinaceous compounds that have antibiotic activity towards pathogenic micro-organisms (virus, fungi, and bacteria amongst others). Antimicrobial peptides make up the essential part of the constitutive and induced plants' defense system. For instance, purothionin was the first AMP isolated from a plant, which has activity against *Xanthomonas phaseoli*, *X. campestris*, *Erwinia amylovora*, *Pseudomonas solanacearum*, *Corynebacterium flaccumfaciens*, *Corynebacterium michiganense*, *Corynebacterium sepedonicum* and *Corynebacterium fascians* (De Caley *et al.*, 1972).

Over 800 peptides have been isolated and studied to date. Most display antimicrobial activity as expected but they also increase the immune system naturally (Giuliani *et al.*, 2007). Research studies have suggested that AMPs have medical importance because of their ability to tackle wide varieties of microbes yet not lead to resistance issues often caused by the antibiotics in current use. Corr *et al.* (2007) carried out the most convincing research study in which they found out that infections caused by *Listeria monocytogenes* in mice were greatly reduced by *Lactobacillus salivarius* UCC118, a bacteria strain that makes bacteriocins. Additionally, research showed that when probiotic milk is given to pigs with diarrhea, the lactic acid bacteria (LAB) strains present in the milk produce bacteriocins that can lessen the seriousness of the infections caused by *Salmonella* (Walsh *et al.*, 2008; Casey *et al.*, 2007).

For instance, in the food industry, chemicals such as benzoates, sorbic acid, nitrites have been the preferred choice for preserving foods. The demand for other source of natural preservatives is rapidly growing due to consumer health concerns, which brought about synthesizing AMPs from foods. Nisin is one of the most studied and commercialized biopreservative, it is an antibacterial peptide that was obtained from certain strains of *Lactococcus lactis* (Lee and Paik, 2016; Rai *et al.*, 2016).

AMPs indeed have important roles in host barrier to fend off attacks from pathogens so they are deemed as super important part of the immune system of the host. With stability to heat and ability to rapid dissolve in water, these peptides have great potentials. Numerous research studies have discovered that AMPs still have minimal activity at extreme environmental conditions such as low pH. At same concentration, AMPs are more active than conventional antibiotics (Meng *et al.*, 2010).

With these characteristics, research into isolation and characterisation of AMPs is an emerging field which is expected to contribute immensely to agriculture, clinical and food industries. The study of AMPs may also open up opportunities for biotechnological exploitation for large-scale production of the over-expressed antimicrobial peptides.

1.3. Problem Statement

Only a mere thirty plant crops are used to feed the world out of the thirty thousands plant species that are edible, though numerous underutilised crops are stable food source to the poorest people around the world (Food and Agriculture Organization, 2017). *Vigna subterranea* is one of the most important indigenous legumes in Africa and Asia and contains high amounts of protein. *V. subterranea* has many mechanisms for withstanding biotic and abiotic stress during its life cycle, one of which is the production of AMPs. The range of the AMPs produced by this plant has however not been investigated or characterised.

This study attempts to clone and characterise several AMPs from *V. subterranea*. It was found that there is a lack of *V. subterranea* sequence information coupled with an apparent lack of AMP sequence conservation, even between closely related species. This study aims to enhance the understanding of sequences and activity of antimicrobial peptides produced by *V. subterranean*, especially as the analysis of gene expression is becoming essential in biological research. The patterns of expressed genes will enable understanding of complex regulatory networks, which will likely enable identifying genes involved in diseases as well as new biological processes.

The study commenced by focusing on apparently conserved regions of AMPs produced by leguminous plants and preparing primers from that region in order to screen the *V. subterranea* genome. Following PCR amplification, sequencing and/or cloning, candidate AMP genes will be further characterised using bioinformatics techniques for early expression patterns during germination.

1.4. Study Aim and Objectives

The main aim of the research study was to amplify and characterise as many *V. subterranea* AMP sequences as possible. To accomplish this aim, the specific objectives were

- a) To design specific primers for the conserved regions of defensin-type leguminous plants.
- b) To amplify by polymerase chain reaction (PCR) and clone defensin gene segments from *Vigna subterranea*.
- c) To characterize the isolated defensin gene fragments with *in silico* methods.
- d) To clone and express the full length *V. subterranea* defensin genes.
- e) To analyse the expression pattern of the gene in *V. subterranea* using reverse transcription quantitative PCR.

1.5. Scope of study

The work was started without the knowledge of the level of sequence conservation of *Vigna subterranea* AMPs and those of other legumes. The thesis focused on isolation and characterization of defensin-type antimicrobial peptides present in *V. subterranea*.

Polymerase Chain Reactions were done with designed defensin specific primers which were optimized at different annealing temperature and magnesium chloride concentration. The isolated peptides will be verified as defensin with sequencing, after which quantitative Reverse Transcription Real Time PCR (RT-qPCR) was used to measure the expression level of defensin mRNA in *V. subterranea* based on the reference gene (β -tubulin) and/or internal control.

1.6. Expected Contribution to Knowledge

The antimicrobial peptides in plants especially legumes have been investigated in many forms and under varying conditions. At the time of writing the author is unaware of much similar research on defensin-type peptides in *Vigna subterranea* having been carried out. The sequence obtained for the peptides is expected to produce important resources that will provide genetic constructs for further possibility of biotechnological utilisation.

1.7. Thesis Outline

In **Chapter 1**, the background and motivation for the project are briefly outlined. The reader is also briefly introduced to the history of *Vigna subterranea* and antimicrobial peptides. The introductory chapter closed with the structure of the thesis.

Chapter 2 reviews composition and cultivation of *Vigna subterranea*. In addition, antimicrobial peptides i.e. defensins that have been isolated from leguminous plants and the heterologous expression systems were discussed.

The molecular techniques used in this study is the focus of **Chapter 3**, wherein amplification and cloning of antimicrobial peptides from *V. subterranea* was done. *In vivo* gene expression analysis of defensin in the plant was also done.

In **Chapter 4**, the results of the isolated peptides are presented and discussed. The chapter is mainly on defensin-type peptides isolated and its characterization *in vivo*.

The thesis is concluded with recommendations and conclusions in **Chapter 5**.

It is followed by a list of references and the appendices.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. *Vigna subterranea*

Vigna subterranea (Bambara groundnut) (Fig. 1) is a grain legume that is grown in the semi-arid zone of sub-Saharan Africa. The old botanical name is *Voandzeia subterranean* (L.) thousands. *V. subterranea* is a member of the Plantea of the family of Fabaceae and subfamily of Faboidea (Beentje 2010).



(a)



(b)

Figure 1: *Vigna subterranean* cultivated via tissue culture (a) and in soil (b) showing the erect growth habit.

2.1.1. Crop cultivation

Vigna subterranea can be cultivated at altitudes of about 1600 m beyond level of the sea. It thrives best in loose, friable top soil at temperature ranges of 20°C to 28°C. Stored *V. subterranea* seeds are viable for one year and the seeds germinate between seven to 15 days. After sowing, flowering starts in 30 to 35 days and may continue till the end of the plant life cycle. *V. subterranean* grows into a full plant within 110 to 150 days with rainfall of 600 - to 700 mm but more rain at harvest time may damage the crop due to the presence of excess water likely to be retained by the soil. The plant requires little to no chemical fertilisers due to its nitrogen fixation properties. *Vigna subterranea* is often cultivated by

resource-poor farmers especially women, who intercrop it with other major crops. The harvesting is done by pulling the plant out of the soil (Ceasar 2007).

2.1.2. Food composition

Vigna subterranea seeds have been reported to contain 42 - 65% carbohydrates, 17 - 25% proteins and 6% lipids. Its energy content has been shown to be much more than others (Mwale *et al.*, 2007). It also contains iron, potassium, fibre, and calcium (Yao *et al.*, 2015). The main amount of amino acid in *V. subterranea* was determined as 32.5g/100g crude protein in comparison to the amount in *Glycine max* (44.70 – 46.50), *Vigna unguiculata* (28.09 - 49.96), *Arachis hypogea* (42.80), *Cajanus cajan* (29.77 – 67.08) and *Phaseolus vulgaris* (41.10) (Aremu *et al.*, 2017).

In African countries, such as Zimbabwe, *Vigna subterranea* is mostly processed into a snack or bread-like product known as *mutakura*, by boiling it in water for a few hours. *Mutakura* is often mixed with groundnut or maize to make a thick porridge or mashed with sweet or Irish potatoes to make *Jambalaya*. To wean infants, people in Pfungwe endorsed a weaning food made with mashed groundnut and *V. subterranea* which further buttress the nutritive values of *V. subterranea* (Mubaiwa *et al.*, 2018).

The medicinal value of *V. subterranea* has been put to good use by the Luo tribe in Kenya, who use a boiled mixture of *V. subterranea* and *Zea mays* to treat diarrhoea. Also, a paste of ground leaves of *V. subterranea* and *Lantana trifolia* L. is being used as an insecticide and to bathe livestock (Mkandawire, 2007). Some of these medicinal values have been found to be possible due to the presence of AMPs (Ajiboye and Oyejobi, 2017).

2.2. Antimicrobial Peptides (AMPs)

Antimicrobial peptides are natural molecules that are biologically active and are important components for cytotoxicity on invading pathogens, made by various organisms. The secondary structures of AMPs range from a loop, disulphide bridges, and α -helices to β -strands. These diverse structures are highly important for the wide range of antimicrobial activity of these peptides (Pushpanathan *et al.*, 2013). AMPs have been isolated from three classes of life, including (a) prokaryotes, such as bacteria (bacillomycin, syringomycin), (b) fungi (aculeacins, leucinostatins), (c) animals, such as fish (misgurin, pleurocidins), mammals (histatin, indolicidin), amphibians (nigrocin 1 and 2, tigerin-1), insects (abaecin, lebecin), echinoderms (centrocins, filamin A), and (d) plants (defensins, thionins, lipid transfer proteins) (Pushpanathan *et al.*, 2013).

2.3. Plant Antimicrobial Peptides

Plant antimicrobial peptides are constitutively expressed by plants to fight microbial infections and belong to the non-specific defense system of the plant. They can be isolated from various parts of the various species of the plant, including the leaves, root, stem, flowers and seeds. Significant features of plant AMPs are their high glycine and/or cysteine content and the presence of disulphide bridges, which are essential to improve the structural stability of the peptides under stressful conditions (Nawrot *et al.*, 2014). The high cysteine content enables generation of compact structures with multiple sulphide bridges that are resistant to proteolytic and chemical degradation. Common characteristics between plant AMPs include molecular forms, amphipathic nature and positive charges which are primarily associated to their antimicrobial activity (Tam *et al.*, 2015). Structures of plant AMPs is mainly based on their typical disulphide bonds formed and cysteine motifs showing the typical Cys pattern with a distinct number of non-Cys residues flanked by the two bordering Cys structures which decide their conserved structural folds so as to play multiple antimicrobial roles (Tam *et al.*, 2015).

The mechanism of plant antimicrobial peptides depends on its cationic charge at physiological pH and its amphipathic structure. The central hypothesis for AMP mode of action relies on its ability to break down the surface of the bacterial cell wall, as cationic peptides accumulate and attaches to the negatively charged molecules like teichoic acid and lipopolysaccharides which is important to membrane structure. This eventually causes cell death due to activation of several pathways. These peptides mostly form membrane pores which cause ions and metabolite leaking in the pathogens, disruption of respiration process, biological polymer formation, followed by cell death (Pelegrini *et al.*, 2011). Classification of AMPs are still being revised, however the current classification (Figure 2) based on homology of amino acid sequences into main families include; cyclotides, lipid transfer proteins, hevein-type protein, puroindolines, thionins, defensins, and snakins (Nawrot *et al.*, 2014).

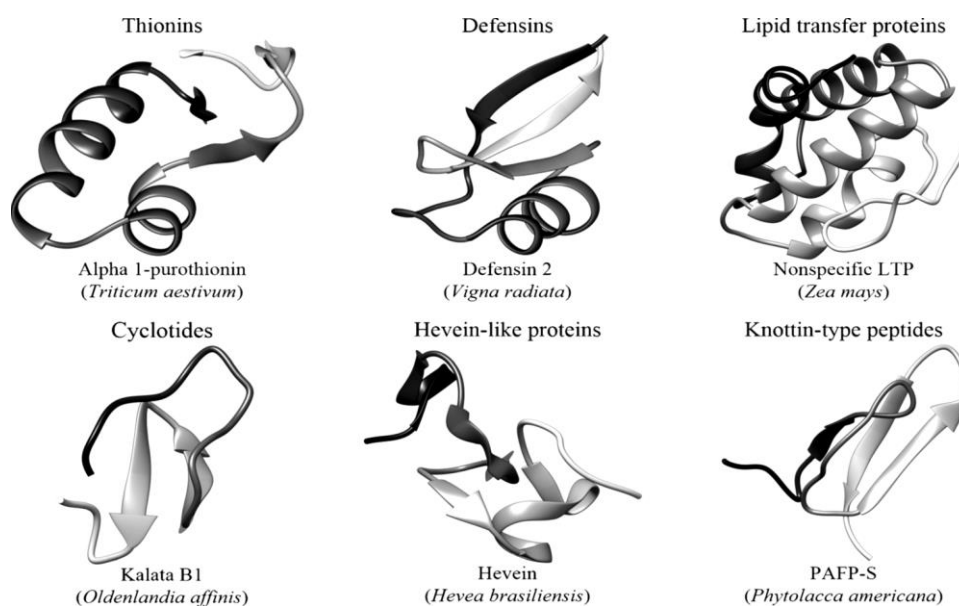


Figure 2: Three Dimensional structures from various families of some antimicrobial peptides (Resource for Biocomputing, Visualization and Informatics, University of California) (Nawrot *et al.*, 2014; Pettersen *et al.*, 2004).

2.3.1. Thionins

Thionins are commonly peptides with size of 5 kDa, with lot of residues i.e. cysteine, lysine, and arginine. The peptides have activity against bacteria, fungi and yeasts. All thionins occur in crucial plant tissues from seeds to leaves. It has been suggested that they exert toxicity through lysis of cell membranes resulting from the toxicity mechanism, which as yet remains unknown. Thionins cause development of pore or unique link with a particular lipid domain due to the direct interaction of the plus charge (thionins) and the minus charge (phospholids) in the fungi's membranes (De Lucca *et al.*, 2005). Thionins have distinct groups, namely α - or β -thionins, but the groups are subdivided into types:

- Type I: also known as purothionins (basic) contain 45 amino acids and four disulphide bridges. It was first isolated from wheat endosperm and has antibacterial effects against *Corynebacterium sepedonicum*, *Erwinia amylovora*, *Corynebacterium flaccumfaciens*, *Xanthomonas phaseoli*, *Corynebacterium fascians*, *Pseudomonas solanacearum*, *Corynebacterium poinsettiae*, *Corynebacterium michiganese*, and *Xanthomonas campestris*. It has also been shown to have antifungal activity against *Rhizoctonia solani* (Nawrot *et al.*, 2014; De Lucca *et al.*, 2005).
- Type II: also known as β -hordothionin and α -hordothionin. They are not as much basic, have 46 - 47 amino acids and four disulphide bridges. They were obtained from *Pyricularia pubera* leaves and nuts (Egorov *et al.*, 2005).

- Type III: have three disulphide bridges and 45 - 46 amino acids. It has been extracted from stems and leaves of species of mistletoe, such as *Viscum album*, *Dendrophthora clavata*, *Phorandendron liga* and *Phorandendron tomentosum* (Stec, 2006).
- Type IV: are neutral in charge, have three disulphide bridges and 46 amino acids. It has been extracted from Abyssinian cabbage seeds (Stec, 2006; Schrader-Fisher and Apel, 1994).
- Type V: are shortened thionins forms commonly found in grains. Hellothionin D was isolated from *Helleborus purpurascens* roots (Stec, 2006; Castagnaro *et al.*, 1994).

2.3.2. Lipid transfer proteins (LTP)

Numerous monocotyledonous and dicotyledonous plants have non-specific lipid transfer proteins (ns-LTPs) and can carry lipids between membranes. They also participate in regulating pools of intracellular fatty acid, biogenesis of membranes, defense against plant pathogens, enabling plants to become suited to new environments, as well as symbiosis, amongst others. The structure of LTPs consists of hydrophobic cavity surrounded by four α -helices which are closely held together by four disulphide bonds. LTP1 (9 kDa) and LTP2 (7 kDa) are the sub families of LTP, which have about 30% similar amino acids (Yeats and Rose, 2008). Only few isoforms of Arabidopsis and rice ns-LTPs have tryptophan. Some LTPs have been shown to have antifungal and antibacterial activity. An nsLTP (9.03 kDa) was found in mung beans (Wang *et al.*, 2004). It was determined to have antibacterial activity against *Staphylococcus aureus*, as well as ability to inhibit fungi's growth i.e. *Fusarium solani*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum* (Egger *et al.*, 2010; Wang *et al.*, 2004).

2.3.3. Cyclotides

Cyclotides are circular proteins that exist naturally and are found in plants, bacteria and animals and have highly similar sequences. Plant cyclotides have a head-to-tail circular backbone, 28 - 37 amino acids and three disulphide bonds intramolecularly arranged in a cyclic cysteine knot (CCK), which is important for cyclotide stability (Craik, 2010). The structure is amphipathic due to the hydrophobic residues that form the surface patch. Cyclotides were extracted from the plant families of *Cucurbitaceae*, *Rubiaceae*, *Violaceae* and *Poaceae* (Gruber *et al.*, 2008). Based on the absence or presence of cis-proline, cyclotides can be classified into two families; namely the bracelets or Mobius. In plants, cyclotides are peptides that are produced by biosynthetic pathways of ribosomes. The preprotein of cyclotides has a signal sequence for endoplasmic reticulum, N-terminal repeat, a pro-region and a domain of cyclotide sequences rather than a short tail (Craik,

2010). Kalata B1 was the first cyclotide to be described which was isolated from *Oldenlandia affinis* (Jennings *et al.*, 2001). In Africa, its main use was to speed up labour and childbirth of women (Mylne *et al.*, 2010). Biological activities of cyclotides include anti-HIV, insecticidal, uterotonic and antimicrobial, amongst others. These characteristics allow the peptides to have potential uses in agricultural and pharmaceutical industries (Craik, 2010).

2.3.4. Defensins

Plant defensins are small (5 kDa) positively charged peptides which are basic, rich in cysteine, 45 - 54 amino acids (Tam *et al.*, 2015). Plant defensins have been reported to have biological activities such as antifungal, proteinase, antibacterial, and activity against amylase (Stotz *et al.*, 2009; Wijaya *et al.*, 2000). Plant defensins have conserved three dimensional structure that include one subunit of α -helix with three β -sheet in parallel joined firmly by four disulphide bridges. They seem to be similar to that of insects and mammals' defense peptides. Pelegrini and Franco, (2005) found that positively charged amino acid residues in defensins enable them to enter the cell membrane which causes destabilisation and disruption of membrane as well as movement of ions. The peptides are classified into four groups depending on their antimicrobial activity, namely group I - alter fungal hyphae and inhibit gram positive bacteria; group II - are not active against bacteria but active against fungi without altering fungi hyphae; group III - are active against bacteria but not active against fungi; and group IV - are active against bacteria and fungi without altering fungal hyphae (Hegedus and Marx, 2013; Carlos *et al.*, 2014). Defensins can be found in bark, tubers, leaves, floral organs, fruits, pods, and roots of plants e.g. Ah-AMP1 from *Aesculus hippocastanum*, Rs-AFP1 from *Raphanus sativus*, Hs-AFp1 from *Heuchera sanguinea*, Dm-AMP1 from *Dahlia merckii* etc. (De Lucca *et al.*, 2005).

Research has revealed cysteine-rich peptides i.e. defensins as significantly characterized within databases which have a multitude of information on numerous plant species (i.e. thirty three), plus *Arabidopsis thaliana*, a model plant and *Oryza sativa*, a crop plant (Silverstein *et al.*, 2007). Gene and genome database analysis have revealed that these genes coding for peptides is about 3% of the entire genome in a plant. *A. thaliana* has been identified to contain at least three hundred genes for defensin (Silverstein *et al.*, 2005).

Mergaert *et al.* 2003 discovered a very huge gene family from *Medicago truncatula* which was characterised by the presence of signal protein, conserved cysteine motifs and small sized genes which coded for small mRNAs (400 – 700 nucleotides) and polypeptides (60 – 70 amino acids). Nineteen out of the forty two *M. truncatula* cDNAs were identified as defensin (DEFL) genes that were induced during nodule development.

2.3.5. Snakins

Snakins are small basic peptides that have been isolated from tubers of *Solanum tuberosum* L (potato). They include cell wall-liked peptides, snakin-1 (StSN1) and snakin-2 (StSN2), which work against lot of fungi and bacteria (Meiyalaghan *et al.*, 2014). Both StSN1 and StSN2 have a length of sixty three amino acids and are 6.9 kDa in size. All snakins have six disulphide bonds and 12 conserved cysteine residues. StSN2 is expressed at locally induced wound sites on plants and exhibit various responses to pathogenic infections, while StSN1 is produced in different parts of the plant during development (Mao *et al.*, 2011; Berrocal-Lobo *et al.*, 2002). They code for proteins with three various domains; recognised signal peptides (18 - 29 residues), a variable region that varies highly in sequence length and amino acids, among family members and a C-terminal region, which covers 12 cysteine residues in about 60 amino acids in a conserved area named GASA domain. Snakin genes participate in plant defense and developmental processes like cell elongation, cell division and flowering (Nahirñak *et al.*, 2012).

2.3.6. Hevein-like protein

Hevein is a 4.7 kDa peptide with lot of cysteine residues, which was first isolated from latex of *Hevea brasiliensis* (rubber tree). In fungi, it hinders hyphal growth but some other Hevein-like proteins have been shown to exhibit antibacterial properties. The chitin-binding domain is in the conserved position of the protein's domain, numerous glycine and cysteine residues are present at that position. The number of sulphide bonds differs among the Hevein-like AMPs. Antifungal activity has been shown by Hevein-like peptides that were isolated from *Triticum kiharae* seeds (WAMP-1a and WAMP-1b). Both seeds exhibited high antifungal activity against *Fusarium oxosporum*, *B. cinerea*, *Fusarium verticillioides*, *Fusarium salani*, *Neurospora crassa* and *Bipolaris sorokiniana*. Antibacterial activity was also shown against *Erwinia carotovora*, *Clavibactermichiganensis* and *Pseudomonas syringae* (Nawrot *et al.*, 2014; Huang *et al.*, 2004).

2.3.7. Puroindolines

Puroindolines isolated from wheat endosperm, are small (13 kDa) basic proteins with five disulphide bridges, which have a distinct domain rich in tryptophan. Pina-D1 and Pinb-D1 genes encode for the two main isoforms PIN-a and PIN-b. The backbone is made up of cysteine residues. The tertiary structure has loops separating four α -helices. Puroindolines exhibit a similar folding pattern like nsLTPs but it has five disulphide bridges while nsLTPs have four. PIN has two additional cysteine, amphiphilic tryptophan-rich domain and monovalent cat-ions (Zhang *et al.*, 2011). PIN-1 makes ion channels in biological and artificial membranes which bind to the cat-ions.

2.4. Defensins in Leguminous Plants

2.4.1. *Lens culinaris* Lc-Def

A novel 5.4 kDa defensin was isolated from the germinating seed of *Lens culinaris* and assayed for antifungal activity. It inhibited 50% growth of *Aspergillus niger* after 48 hours at a concentration of 28.7 μM. The N-terminal sequence of Lc-Def was found to be KTCENLSDSFKGPCIPDGNCNKHCKEKEHLLSGRCRDDFRCWCTRNC. Homology of the protein to defensin was determined by BLAST search in NCBI protein database. Lc-Def was found to have 47 amino acid residues with eight cysteine-forming disulphide bridges (Finkina *et al.*, 2008).

2.4.2. *Medicago truncatula* MtDef1.1 and *Medicago sativa* MsDef1.1 – 1.6

Medicago truncatula is a model legume in genomics to study symbiotic relationships between plants and microorganisms (Rose, 2008). Sixteen genes of the genomic family were discovered while screening the gene index of *M. truncatula* in The Institute for Genomic Research (TIGR). Ectopic overexpression in transgenic potato made the plant resistant to *Verticillium dahliae*, which is a fungal pathogen. A 318 bp PCR product (*MtDef1.1*) was obtained when primers (5' ATGGAGAAGAAATCACTAGC 3' and 5' TTAACATCTTTTAG TACACC 3') were used to amplify *M. truncatula* genomic DNA (Hanks *et al.*, 2005). However, six PCR products about 219 - 793 bp (*MsDef1.1 – 1.6*) were obtained when Genomic DNA of *Medicago sativa* v LegenDairy was amplified. The genes encode a mature protein (45 aa) with a putative signal peptide (27 aa). A 99 bp intron was found to be present in all except in *MsDef1.2*. The expression analysis showed that defensin genes are produced in different tissues of *M. truncatula* during normal growth as well as in response to biotic and abiotic stimuli (Hanks *et al.*, 2005).

2.4.3. *Phaseolus vulgaris* peptide

A 5.4 kDa novel peptide was obtained from *Phaseolus vulgaris* cv. seeds (Brown kidney Bean), where 6.2 mg of the peptide was obtained from 60 g of the beans after purification with three steps (affinitychromatography, FPLC-cation exchangechromatography and FPLC-gel filtration). It showed inhibitory activity against *Mycosphaerella arachidicola* (IC₅₀ value of 3 μM), *Setosphaeria turcica* and *Bipolaris maydis* (Chan *et al.*, 2012). The sequence of the N-terminal, KTCENLADTYKGPCFTTGSCDDHCK, showed high homology to defensins in NCBI protein database. A BLAST of the protein sequence revealed that the sequence was conserved in the gamma-thionins family, which is a group of plant defensins (Chan *et al.*, 2012).

Wong and Ng (2005) isolated a 7 kDa peptide, Vulgarinin, from *Phaseolus vulgaris* cv. (Haricot Bean). It showed activity toward fungi such as *Physalospora piricola*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Mycosphaerella arachidicola*, as well as activity toward these bacteria: *Proteus vulgaris*, *Bacillus subtilis*, *Mycobacterium phlei* and *Bacillus megaterium*.

A 7.3 kDa peptide was isolated from *Phaseolus vulgaris* cv. (Spotted Bean). It showed activity against fungi i.e. *Mycosphaerella arachidicola* (1.8 μ M IC₅₀ value) and *Fusarium oxysporum* (2.2 μ M IC₅₀ value). The peptide also had anti-proliferative activity by repressing the incorporation of [methyl-³H]-thymidine into Leukaemia L1210 cells (IC₅₀ value of 4 μ M) and MBL2 cells (IC₅₀ value of 9 μ M). The N-terminal of the peptide, KYCENLADTYKGPYFTTGSHDDHYKNKEHLRSGRMRDDFF was found to show high homology to defensins in NCBI protein database (Wang and Ng, 2007).

2.4.4. *Phaseolus lunatus* defensin-like peptide

A novel 6 kDa peptide, that has KTCENLADYYRGPCFATSNCDDHCKNKEHLLSGRCRDDFRCWCTRNC at N-terminal, was found in *Phaseolus lunatus* (Lima Bean). It displayed inhibitory activity on growth of *Mycosphaerella arachidicola*, *Physalospora piricola* and *Fusarium oxysporum* as well as Reverse Transcriptase of HIV-1 (Wang and Ng, 2006).

Lunatusin is a 7 kDa peptide that has N-terminal KTCENLADTFRGPCFATSNC, was found to show high homology to defensins isolated from *Phaseolus lunatus* L. (Chinese Lima Bean). It displayed inhibitory activity on growth of *Botrytis cinerea*, *Mycosphaerella arachidicola* and *Fusarium oxysporum* with 2.6 μ M, 0.32 μ M and 1.9 μ M IC₅₀ value respectively. Lunatusin also showed antibacterial activity against *Proteus vulgaris*, *Bacillus megaterium*, *Mycobacterium phlei* and *Bacillus subtilis* at IC₅₀ value of 81 μ M, 115 μ M, 96 μ M and 98 μ M respectively. Lunatusin was also found to inhibit increase of cells for breast cancer (5.71 μ M IC₅₀ value) and also reduce action of Reverse Transcriptase of HIV-1 (120 μ M IC₅₀ value) (Wang and Ng, 2005).

2.4.5. *Trigonella foenum-graceum* Tfgd1

A novel defensin of molecular weight 8.4 kDa was isolated from leaves of *Trigonella foenum-graceum* and expressed in *Escherichia coli*. When assayed for antifungal activity, it inhibited mycelial spread and growth of the fungi, *Phaeoisariopsis personata* and *Rhizoctonia solani* after 48 hours at a concentration of 100 μ M. *Tfgd1* precursor has a coding region of 74 amino acid residues with eight cysteine-forming disulphide bridges of which 27 of the amino acids for a signal peptide. ATCENLADTFRGPCFGNSNCN

FHCKTKEHLLSGRCRDDFRCWCTKRC was found to be N-terminal of the peptide which showed high homology to defensins in UniProt data base (Olli and Kirti, 2006).

2.4.6. *Phaseolus angularis* defensin-like peptide

A peptide with molecular weight (MW) of 7.1 kDa was isolated from *Phaseolus angularis* (big Nepalese red beans) which showed inhibitory activity on growth of mycelial in *Mycosphaerella arachidicola* (1.4 μM IC_{50} value) and *Fusarium oxysporum* (1.8 μM IC_{50} value) (Ma *et al.*, 2009).

2.4.7. *Vicia faba* Limenin

A 6.5 kDa peptide, Limenin was isolated from *Vicia faba* (Shelf Bean). It showed an antifungal activity against *Botrytis cinerea* (IC_{50} value of 2.9 μM), *Fusarium oxysporum* (IC_{50} value of 2.1 μM) and *Mycosphaerella arachidicola* (IC_{50} value of 0.34 μM). Limenin was also found to increase the addition of [methyl- ^3H]-thymidine into mouse splenocytes but represses the incorporation into Myeloma (M1) cells and Leukaemia (L1210) cells (IC_{50} value of 4 μM) and MBL2 cells (IC_{50} value of 9 μM). It showed antibacterial activity against *Proteus vulgaris*, *Mycobacterium phlei*, *Bacillus subtilis* and *Bacillus megaterium* at IC_{50} value of 81 μM , 96 μM , 112 μM and 102 μM respectively. The N-terminal of the peptide which showed high homology to defensins was found to be, KYCENLADTYKGPCFTTG CDDHCKNKEHLLSGRCRDDFRCWCTRNC was found to be (Wong and Ng, 2006).

2.4.8. *Phaseolus coccineus* Coccinin and Phaseococcin

A 7 kDa peptide, Coccinin was isolated from *Phaseolus coccineus* cv major (Large Scarlet Runner Bean). It showed broad spectrum antifungal activity against mycelial growth of *Rhizoctonia solani*, *Physalospora piricola*, *Mycosphaerella arachidicola*, *Coprinus comatus*, *Fusarium oxysporum*, and *Botrytis cinerea* at IC_{50} values of 134 μM , 89 μM , 75 μM , 122 μM , 81 μM , and 109 μM respectively. Coccinin was also found to have antiproliferative activity by repressing the incorporation of [methyl- ^3H]-thymidine into Leukaemia L1210 cells (IC_{50} value of 40 μM) and HL60 cells (IC_{50} value of 30 μM). The peptide showed antibacterial activity against *Bacillus megaterium*, *Mycobacterium phlei*, *Proteus vulgaris* and *Bacillus subtilis* and at IC_{50} values of 102 μM , 96 μM , 81 μM , and 112 μM respectively. Coccinin has also been shown to lessen the functioning of Reverse Transcriptase for HIV-1. KQTENLADTY was found to be N-terminal of Coccinin which showed high homology to defensins (Ngai and Ng, 2004).

A peptide with MW of 5.4 kDa, Phaseococcin was isolated from *Phaseolus coccineus* cv minor (Small Scarlet Runner Bean). It showed broad spectrum antifungal activity against mycelial growth of *Fusarium oxysporum*, *Physalospora piricola*, *Coprinus comatus*, *Mycosphaerella arachidicola*, *Rhizoctonia solani* and *Botrytis cinerea* at IC_{50} values of

89µM, 95 µM, 113µM, 70 µM, 140 µM, and 102 µM respectively. Phaseococcin was found to have antiproliferative activity by repressing the incorporation of [methyl-³H]-thymidine into Leukaemia L1210 cells (IC₅₀ value of 30.2 µM) and HL60 cells (IC₅₀ value of 40.5 µM). The peptide showed ability to hinder bacteria's growth i.e. *Bacillus cereus*, *B. megaterium* and *B. subtilis* at IC₅₀ values of 289µM, 127µM and 350µM, respectively. Phaseococcin also reduces the activity of reverse transcriptase for HIV-1. The N-terminal KCTENLADTYKGPPFFTTG showed high homology to defensins (Ngai and Ng, 2005).

2.4.9. *Gymnocladus chinensis* Gymnin

A 6.5 kDa peptide, Gymnin was isolated from *Gymnocladus chinensis* Baill (Yunnan Bean). Research showed that it inhibit *Mycosphaerella arachidicola* (10 µM IC₅₀ value) and *Fusarium oxysporum* (2 µM IC₅₀ value). Gymnin was also found to increase the addition of [methyl-³H]-thymidine into mouse splentocytes but was shown to repress incorporation into three tumour cell lines i.e. Leukaemia (M1 and L1210) cells and Hepatoma (HepG2) cells. It also lessens the activity of Reverse Transcriptase for HIV-1 (200 µM IC₅₀ value). The peptide's N-terminal with high homology to defensins, was found to be KYCENLADDY (Wong and Ng, 2003).

2.4.10. *Tephrosia villosa* TvD1

An 8.2 kDa peptide, TvD1 was isolated from herb, *Tephrosia villosa*. A Reverse Transcriptase - PCR product of 228 bp was obtained when primers 5' GGGTACCATGGAGAAGAAATCACTAGC 3' and 5' GGGATCTTTAACAATCTTTTAGTACACCA 3', were designed from alfalfa defensin and used to amplify the clone and the cDNA of TvD1. The gene encodes for 75 aa peptide, with the N-terminal of the peptide found to be KYCENLADTYRGPCFTTGSCDDHCKNKEHLLSGRCRDDFR. This showed high homology to defensins in the NCBI protein database. The first 28 aa showed a strong protein as signal peptide. TvD1 was found to have antifungal activity against *Phaeoisariopsis personata* (IC₅₀ value <10 µM) as well as for *Curvularia* sp, *Fusarium oxysporum*, *Botrytis cinerea*, *Alternaria helianthi* and *Fusarium moniliforme* at an IC₅₀ value of <25 µM (Viyajan *et al.*, 2008).

2.4.11. *Vigna angularis* VaD1

A 5.2 kDa peptide, VaD1 was isolated from *Vigna angularis* (Azuki Bean), where a 228 bp of RT-PCR product was obtained when primers (5' ACCTCAACAATTCATCACTCATG 3' and 5' TAGCAGTGATGCTGCTATATTTATTTGTGA 3') were used to amplify *V. angularis* DNA. The gene codes for peptide with 46 amino acids. VaD1 was found to have antifungal activity against *Trichophyton rubrum*, *Fusarium oxysporum* sp. *pisi*, and *Fusarium oxysporum* with IC₅₀ values of >500 µM, 53.2 µM and 30 µM respectively. The peptide was

also found to have antibacterial activity against *Xanthomonas campestris* pv. *Vesicatoria*, *Bacillus cereus*, *Staphylococcus epidermis*, *Escherihia coli*, *Salmonella typhimurium*, *Erwinia carotovora* v. *Carotovora*, *Pseudomonas syringae* pv. *Syringae*, *Proteus vulgaris* and *Salmonella enteritidis* with IC₅₀ values of 40.8 µM, >500 µM, 36.6 µM, >500 µM, 143.4 µM, 1000 µM, >1000µM, >500 µM, >1000µM, and >1000µM respectively. The N-terminal of the peptide was found to be KTCMTKKEGWGRCLIDTTCASCRKQGYKGGNCKGMRRTCyclLDC, with high homology to defensins in NCBI protein database (Chen *et al.*, 2005).

2.4.12. *Pisum sativum* Psd1

A 5.1 kDa peptide, *Psd1* was isolated from *Pisum sativum* (Garden pea). It was found to have antifungal activity against *Aspergillus niger*, *Neurospora crassa*, *Fusarium solani* and *Aspergillus vesicolor* with IC₅₀ values of 12.1 µM, 11 µM, 5.3 µM and >100 µM, respectively. A 160 bp of RT-PCR product was obtained when primers (5' CTCGAGAAAAGAAAGACTT GCGAACACTTAGCTGACACCTACAGGGGAGTATCTTCACG 3' and 5' GGAATTCCTAA CAGTTTTGAGTACAGAA ACACTTCCAGTTGTGACA 3') were used to amplify the cDNA of PsD1. The gene codes for mature protein of 46 aa residues. The N-terminal was found to be KTCEHLADTYRGVCFNASCDDHCKNKAHLISGTCHNWKCFCTQNC with high homology to defensins in UniProt database (Cabral *et al.*, 2003; Almeida *et al.*, 2001).

2.4.13. *Cicer arietinum* YZDCM

A novel 5.4 kDa defensin was isolated from seeds of *Cicer arietinum* (Chickpea). The N-terminal of the peptide was found to be ACCENLADTYRGPCF which showed high homology to defensins in UniProt database (Yili *et al.*, 2012).

According to Parween *et al.* (2015), a novel defensin of 8.4 kDa was isolated from seeds of *Cicer arietinum* (Chickpea), with N-terminal of the peptide found to be ARCENLADTYRGPCFTTGSCDDHCKNKEHLVSGR CRDDFRCWCTKNC which showed high homology to defensins in UniProt database.

2.4.14. *Cajanus cajan* Defensin

Research performed by Olli and Kirti (2003), showed a peptide with molecular size of 8.1 kDa and N-terminal of the peptide found to be MEKKSLAGLCFLFLVLFVAQEIVVTEAKTCENLADKYRGPCFSGCDTHCTTKEHAVSGR CR, was isolated from *Cajanus cajan* (Pigeon pea).

2.4.15. *Arachis diogoi* Defensin

A novel 8.4 kDa peptide was isolated from *Arachis diogoi* (Wild peanut), with N-terminal of the peptide, ATCENLADTFRGPCFGNSNCNFHCKTKEHLLSGRCRDDDFRCWCTKRC showing high homology to defensins in UniProt database (Olli and Kirti, 2002).

2.5. Heterologous Expression Systems (HES) for Plant Defensin

Plant defensins have very low levels of endogenous expression which could make purification of these defensins difficult. This is what necessitates the use of Heterologous Expression Systems (HES) which are used for large-scale production of purified active defensins since isolation from natural sources yield low quantities and can be time consuming and labour intensive (Gazzaneo *et al.*, 2017).

2.5.1. Bacteria

The most commonly used microorganism for heterologous protein production is *Escherichia coli*, as it grows quickly, has been characterised and its genetics and numerous compatible expression vectors with well know cloning protocols are widely available and has been extensively studied. However it has some limitations which have to be overcome to accomplish effective protein production. Firstly, the toxicity of AMPs to host strains must be avoided by preventing the natural activity of the AMP. Secondly, the size and chemical structure of the AMP makes it unstable and a target for proteases made by host cells. Some of the approaches to handle these include using certain engineered *E. coli* strains (BL21, C41, pLysS Origami and Rosetta), vectors bearing a N-terminal *pepB* signal, carrier proteins and secretion signals (thioredoxin reductase and glutathione-S transferase) and finally using aggregation-inducer carriers to promote formation of inclusion bodies (Gazzaneo *et al.*, 2017; Parachin *et al.*, 2012). Examples of plant defensin produced in *E.coli* are listed below:

2.5.1.1. PpDFN1 peptide from *Prunus persica*

Specific primers were used to amplify cDNA coding for mature PpDFN1 peptide in total RNA isolated from leaf, fruit skins and whole flower of *Prunus persica*. Using *Hind*III and *Bsp*HI, the PCR product was ligated into the pET-32 vector (Novagen) and subcloned into plasmid (PET32-*Ppdfn1*). *Escherichia coli* BL21 (DE3) Origami pLys cells (Novagen) were transformed with the resultant vector. Expression was initiated with Isopropyl β -D-1-thiogalactopyranoside (IPTG). The purified peptide was found to prevent growth of *Monilinia laxa*, *Penicillium expansum* and *Botrytis cinerea* with IC₅₀ values of 9.9, 1.1 and 15.1 μ g/mL, respectively (Nanni *et al.*, 2012).

2.5.1.2. Defensin from *Pinus sylvestris*

A PCR amplicon of 174 bp resulting from the amplification of cDNA encoding *Pinus sylvestris* (Scots pine) defensin 1 was digested with *EcoR1* and *NcoI*, then ligated into vector pET42a (Novagen). The recombinant plasmid was transformed into *Escherichia coli* BL21 cells. The purified peptide, PsDef1 of 5.6 kDa was found to have activity against *Fusarium oxysporum*, *Fusarium solani*, *Heterobasidion annosum*, and *Botrytis cinerea* with IC₅₀ values of 4.0, 1.4, 0.7 and 2.6 µg/mL, respectively (Kovaleva *et al.*, 2011).

2.5.1.3. Defensin with snakin from *Solanum tuberosum*

PCR products obtained after amplification of coding region of *Solanum tuberosum* snakin-1 and defensin-1 were cloned into PCR[®] II TOPO Vector[®] (Invitrogen[™]). Expression cassettes were made and used to transform *E.coli* cells because the vector has N-terminal pelB signal sequences which simplifies localisation to the periplasm and reduces insoluble fractions during purification. The peptides were found to have strong activity against *Clavibacter michiganensis* subsp. *Sepedonicus* with IC₅₀ values of >1.5 µM, *Botrytis cinerea* and *Colletotrichum coccoides* with IC₅₀ values >5 µM but it didn't really inhibit the growth of *Pseudomonas syringae* pv. *tabaci* and *Pseudomonas syringae* pv. *syringae* (Kovalskaya and Hammond, 2009).

2.5.2. Yeast

Studies have shown that yeast species are becoming the best alternative for expression of defensins (Cregg *et al.*, 2000; Parachin *et al.*, 2012; Vianna *et al.*, 2012). *Pichia pastoris* showed some advantages over *E. coli* due to increases in solubility and quantities of expressed proteins as well as posttranslational modifications (i.e. glycosylation). Additionally, *P. pastoris* requires less purification steps since it tolerates secretion of the recombinant protein, which enables scale-up processes because it does not require a carrier protein like *E. coli* (Gasser *et al.*, 2013). Since *P. pastoris* does not produce toxic waste, it can be grown to a high cell density with economical culture media. However it needs creation of many strains and promoters to obtain a higher yield of protein (Gazzaneo *et al.*, 2017). Examples of plant defensin produced in *P. pastoris* are listed below:

2.5.2.1. rPsd1 from *Pisum sativum*

Primers were used to amplify the cDNA obtained from wet pea seeds. A 160 bp amplicon was firstly ligated into pGEM[®]-T easy vector, then ligated into pPIC9 vector and used to transform *E. coli* JM109 cells. The linear plasmid was electro-transformed into the yeast, *Pichia pastoris*. The purified recombinant peptide rPsd1 was found to have almost the same

antifungal activity (IC_{50} = 16.6 μ g/mL) as the original peptide (IC_{50} = 12.1 μ g/mL) (Cabral *et al.*, 2003).

2.5.2.2. VrD1 from *Vigna radiata* (mung bean)

Total cellular DNA from mung bean was amplified with primers made according to VrCRP nucleotide sequences. Obtained PCR product was ligated into pGEM[®]-T easy vector and sequenced. This was then used as a template for amplification reaction in order to obtain a 154 bp amplicon which codes for mature VrD1 was ligated into the pPIC9K vector and transformed into *E. coli* cells. The recombinant was purified to ensure that the VrD1 fragment has the accurate reading frame. Linearized plasmid was integrated into a specific locus in genome of *P. pastoris*, and electro-transformed into competent yeast cells (Chen *et al.*, 2004).

2.5.3. Plants

Due to the antimicrobial activity of AMPs, their expression is hindered in microorganisms, despite the development of methods such as controlled transcription and pro-peptide fusions. On the other hand, expression systems in plants have been created as an alternative means for production of peptides which do not require controlled expression or sophisticated processing and folding pathways. Therefore, plants are also a noteworthy choice for the expression of many families of AMPs (Parachin *et al.*, 2012). Examples of such AMPs are below:

2.5.3.1. MsDef1 defensin in Tomato

In order to make transgenic tomato plants expressing MsDef1 transgene, particle accelerating mediated transformation was done using pGLDef1 vector. PCR analysis detected a 250 bp band of MsDef1 which confirms the integration of the gene. Multiple insertion copy numbers for most transgenic tomato genomic DNA was revealed by Southern blot hybridization. Abdallah *et al.* (2010) determined that the average *Fusarium* wilt resistance percentage was 52.4% of all transgenic plants undergoing *Fusarium oxysporum* treatment while the degree of resistance varied among the transgenic lines.

2.5.3.2. Wasabi defensin in *Colocynthis citrullus* L. (Egusi melon)

Agrobacterium tumefaciens EHA101 containing a shuttle vector was synthesized from *Wasabi japonica* and used to inoculate young explants of 'Egusi' varieties. Amplification reactions confirmed that fragments of defensins exist in the transgenic Egusi lines. Genomic DNA of the transgenic clones was digested with restriction enzyme, XbaI and used for southern hybridization which revealed the integration of genes in the Egusi genome

with copy number varying from one to five. Additionally, Northern hybridization revealed the accumulated transgene transcript in various transgenic plant lines. When transgenic Egusi plants were infected with *Alternaria solani* and *Fusarium oxysporum*, lesions formed on small spots at the infection area which was less than 20% of the entire leaf area and other disease symptoms on leaves developed slowly, showing seven days after inoculation. Furthermore, transgenic Egusi lines producing this gene lived for extra 14 days on media containing 1 mL fungal suspension, which showed high resistance to *F. oxysporum* infection (Ntui *et al.*, 2010).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Molecular Biology Approaches / Methods

In this chapter, details of all the relevant procedures are presented (Figure 3). DNA was isolated from leaves of *Vigna subterranea* using cetyl trimethylammonium bromide (CTAB) method. The target defensin gene was amplified with designed primers using a Bio-Rad T100 Thermal Cycler. The amplicon was cloned and sequenced. Phylogenetic analysis was done to analyse the nucleotide and amino acid sequences of the resultant gene. *In vivo* gene expression profiling was done to do absolute and relative quantification of defensin gene in *Vigna subterranea*.

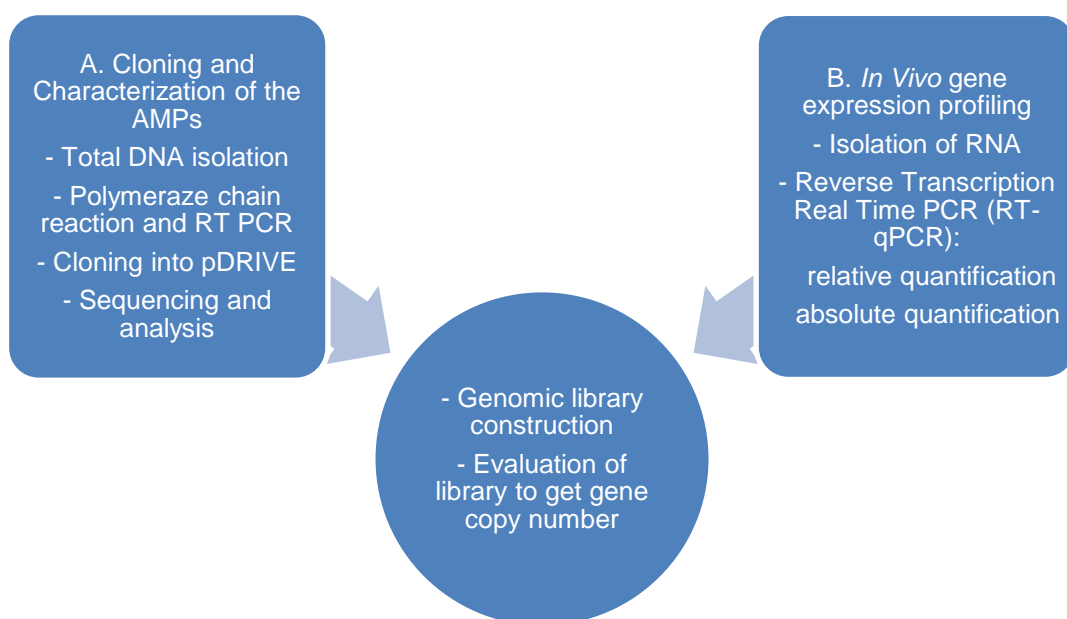


Figure 3: Showing flow diagram of the molecular biology methods used to isolate and characterise defensin gene in *Vigna subterranea*.

3.1.1. DNA Isolation

Genomic DNA was isolated from *Vigna subterranea* using the modified cetyl trimethyl ammonium bromide (CTAB) protocol (Allen *et al.*, 2006). The leaf sample (0.1 g) was weighed, frozen at 80 °C and ground using a sterile frozen pestle and mortar. The powder was scooped into 4 x 1.5 mL micro centrifuge tube. CTAB buffer (500 µL) and β-mercaptoethanol (70 µL)

were added to each of the micro centrifuge tubes and incubated for 20 minutes at 55 °C in a recirculating water bath before centrifuging the tubes at 12000 rpm for 5 minutes. The supernatant was pipetted into sterile micro centrifuge tubes, four hundred and fifty micro litres of chloroform was pipetted into each tube and mixed through inversion before the tubes were centrifuged at 12000 rpm for 5 minutes. The supernatant (aqueous phase) was then pipetted into a clean micro centrifuge tube. Three hundred micro litres of 7.5 M ammonium acetate and two hundred and seventy micro litres of isopropanol was then added to the supernatant and mixed by inverting the tubes. These were stored at -20 °C for an hour and centrifuged at 12000 rpm for 5 minutes to form a pellet. The supernatant was removed. Five hundred micro litres of ice cold ethanol (70%) was added to wash the pellet prior to further centrifugation at 13000 rpm for 5 minutes. The supernatant was removed and the DNA pellet was left to dry for about 15 minutes. The DNA pellet was re-suspended in sixty micro litres of sterile distilled water and then incubated at 65 °C for 20 minutes in a recirculating water bath to aid suspension. The concentration of the DNA was determined using Thermo Fisher Scientific NanoDrop 2000 spectrophotometer. Quality and presence of DNA was visually analysed by agarose electrophoresis, where ten micro litres of the DNA was analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

3.1.2. Primer Design

Multiple sequence alignment of various plant AMPs was done using BLAST on NCBI website to determine the conserved regions of various defensin-type plant antimicrobial peptides. Specific primers were designed and evaluated to target the genes of interest (VsDef P1, VsDef P2, VsDef P3, VsDef P4 and VsDef P5). The plants were propagated by tissue culture on a growth medium containing Murashige and Skoog basal medium (Merck, Germany), to aid fast growth and development.

The primers were ordered from Inqaba Biotech, South Africa and evaluated by BLAST with the genome sequences of *Vigna unguiculata* and *Cicer arietinum* to check how the primers would amplify desired sequence in *Vigna subterranea* genome. The primers (Table 1) were used in cloning, PCR, cDNA synthesis and RT-PCR.

NOTE:

- VsDef P1 and VsDef P2 are primer pairs designed to target consensus sequences of defensin genes from *Cicer arietinum*, *Medicago truncatula*, *Trigonella foenum-graceum*, *Medicago sativa* and *Datura stramonium* (Appendix A1.1).
- VsDef P3 is a primer designed from consensus sequences of defensin genes from *Arachis diogeni*, *Trigonella foenum-graceum*, *Cicer arietinum*, *Vicia faba* and *Pisum sativum* (Appendix A1.2).
- VsDef P4 is a primer designed from consensus sequences of defensin genes from *Vigna unguiculata*, *Vigna angularis var. angularis*, *Phaseolus vulgaris*, *Vigna radiata var. radiata* and *Cajanus cajan*. When the sequence of *Vigna unguiculata* defensin was analysed, it was shown to be similar to that of *Vigna subterranea*, so new primers (VsDef P5) were designed to target the full defensin gene. (Appendix A1.3).

Table 1: Sequences of primers designed from conserved regions of defensins from leguminous plants

Name	Sequence	Length	GC content (%)	Tm (°C)	Expected amplicon size
VsDef P1 F1	5' – GCG AGG TGT GAG AAT – 3'	15 mer	53.33	46	211 bp
VsDef P1 R1	5' – ATT CTC ACA CCT CGC - 3'	15 mer	53.33	46	
VsDef P2 F1	5' – ATG GAC AAG AAA TCA CTA GC - 3'	20 mer	40	56	217 bp
VsDef P2 R1	5' – TTA ACA ATT TTT GGT GCA CC – 3'	20 mer	35	54	
VsDef P3 F1	5' – TCA CTA GCT GGG TTG TGC TT – 3'	20 mer	50	60.4	123 bp
VsDef P3 R1	5' – ACA GCC ACT GAA GCA TGG TC – 3'	20 mer	55	62.45	
VsDef P4 F1	5' – TGG TAA GCC TTT CTC CAC GC – 3'	20 mer	55	62.45	282 bp
VsDef P4 R1	5' – CGC TGA AAC GTT CAG TTC GG – 3'	20 mer	55	62.45	
VsDef P5 F1	5' – ATG GCT CGC TCT GTG C – 3'	16 mer	62.50	56.46	397 bp
VsDef P5 R1	5' – TTA ACA GTG TTT GGT GC – 3' GCACCAAACACTGTAA	17 mer	41.18	49.79	

3.1.3. Polymerase Chain Reaction (PCR) Assay

3.1.3.1. Annealing Temperature (Ta) Gradient PCR

Annealing Temperature Gradient PCR was done using a Bio-Rad T100 Thermal Cycler to optimise the designed primers. Table 1 shows the specific primers that were used. The reaction was done in a 25 μ L reaction volume which contained 1x Taq Ampliqon II Master Mix, 2 mM MgCl₂, 0.2 μ M F1 forward primer, 0.2 μ M R1 reverse primer, 171.2 ng of *V. subterranea* template DNA sample and sterile distilled water to adjust volume to 20 μ L. The amplification processes started with a preliminary denaturation step at 95 °C for 5 minutes, followed by 34 cycles of 95 °C for 30 seconds, 43 – 65 °C for 30 seconds and 72 °C for 30 seconds. The reaction was concluded with a final extension at 72 °C for 5 min, followed by cooling and storage at 4 °C. The quality of the PCR amplicon was visually analysed by agarose electrophoresis, where ten micro litres of the amplicon was analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

3.1.3.2. MgCl₂ Gradient PCR

Magnesium chloride gradient PCR was done using a Bio-Rad T100 Thermal Cycler to optimise the designed primers, as shown on Table 1. A 25 μ L reaction containing 1x Taq Ampliqon II Master Mix, 2 mM MgCl₂, 0.2 μ M forward primer, 0.2 μ M reverse primer and 207.3 ng of *V. subterranea* template DNA sample and sterile distilled water to top up volume to 25 μ L was set up. The amplification processes started with a preliminary denaturation step at 95 °C for 5 minutes, followed by 34 cycles of 95 °C for 30 seconds, specific annealing temperatures [40 °C (VsDef P1), 47.2 °C (VsDef P2 and VsDef P3), 51.3 °C (VsDef P4) and 49.1 °C (VsDef P5)] for 30 seconds and 72 °C for 30 seconds. The reaction was concluded with a final extension at 72 °C for 5 min, followed by cooling and storage at 4 °C. The quality of the PCR amplicon was visually analysed by agarose electrophoresis, where ten micro litres of the amplicon was analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

3.1.4. PCR Cleanup

Cleanup of PCR amplicon was done using the Nucleospin® Gel method according to the manufacturer's protocol (Macherey-Nagel, Germany). The volume of the PCR amplicon product was adjusted to 100 μ L with sterile distilled water. Buffer NT1 (200 μ L) was added to the tube. The Nucleospin® gel column was placed in a sterile 2 mL collection tube and 700 μ L of the sample was loaded and centrifuged at 13000 rpm for 30 seconds in order to bind the DNA to the column. The flow through was thrown out and the column was placed back into the collection tube. Buffer NT3 (700 μ L) was pipetted into the column which was then

centrifuged at 13000 rpm for 30 seconds to wash the membrane. The flow through was thrown out and the column was placed back into the collection tube and centrifuged at 11000 g for 1 minute to let the membrane dry. The column was then placed in a new 1.5 mL tube. Thirty μL of NE Buffer was added to the column and incubated at room temperature for 1 minute. The column was centrifuged at 13000 rpm for 1 minute to elute the DNA. The concentration of the DNA was determined using Thermo Fisher Scientific NanoDrop 2000 spectrophotometer. The quality of the cleaned PCR amplicon was visually analysed by agarose electrophoresis, where ten micro litres of the amplicon was analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

3.1.5. Ligation

Ligation was done using the Qiagen® PCR cloning kit (Qiagen, United States). Prior to setting up the reaction mixture, pDrive cloning Vector DNA and 2 x Ligation Master Mix were thawed on ice. To prepare the ligation-reaction mixture, 4 μL of PCR amplicon, 1 μL of pDrive DNA and 5 μL of 2 x Ligation mix were pipetted into a tube. A pipette tip was used to briefly mixed the content of the tube which was then placed in a fridge at 4°C for 30 minutes. The tube was stored at -20 °C until further use.

3.1.6. Preparing Competent Cells with Calcium Chloride

A culture of *Escherichia coli* strain JM109 was thawed and streaked on Luria Bertani (LB) agar plates and incubated for 16 hours at 37 °C. One colony from the streak plate was inoculated into 9 mL LB broth in a test tube as a starter culture and placed in a shaking incubator for 16 hours at 37 °C. One milli liter from the broth culture was inoculated into 2 x 200 mL LB broth in 1 L flasks and incubated for at least 3 hours at 37 °C with vigorous shaking. The rate of growth of the culture was frequently checked by measuring the Optical Density (OD) with a spectrophotometer every hour until OD₆₀₀ of 0.4 was reached. The cells were aseptically removed from the flasks into sterile ice cold 250 mL polypropylene tubes. The culture was cooled on ice for 10 minutes. Centrifugation at 2700 g for 10 minutes at 4 °C was done to recover the cells. The supernatant was thrown out. The tubes were inverted on a pad of paper for ease of drainage of remaining medium. Thirty milli liters of cold 80 mM MgCl₂ – 20 mM CaCl₂ solution was measured into each tube to suspend the pellet. The cells were recovered by centrifugation at 2700 g for 10 minutes at 4 °C. The supernatant was decanted from the pellet. The tubes were inverted on a pad of paper. Eight milli liters of ice cold 0.1 M CaCl₂ solution was added to each tube to suspend the pellet. Competent cells (300 μL) were pipetted into sterile chilled 1.5 mL micro centrifuge tubes. Thirty percent glycerol was added to each tube to prevent settling of the cells and stored at -70 °C until further use.

3.1.7. Transformation

The prepared competent cells were thawed on ice and LB medium was thawed at room temperature. Two micro liters of ligation-reaction mixture was added to a tube containing 300 μ L of chemically competent cells and mixed gently. The tube was kept on ice for half an hour and heated in a waterbath at 42 °C for 30 seconds. The tube was then chilled on ice for 2 minutes. Five hundred micro liters of LB medium was added to the chilled tube to resuscitate the cells. Then Two hundred micro liters of the transformed cells were plated on LB agar spread plates containing ampicillin, X-gal and IPTG. Empty vector DNA was also plated to serve as control. The plates were incubated at 37 °C for 16 hours. For blue/white screening, the plates were further incubated at 4 °C for a few hours.

After the cloning experiments, Plasmid DNA was isolated from 10 white colonies on the plates to identify possible recombinant strains for sequencing and further analysis.

3.1.8. Screening for recombinants - Plasmid DNA isolation

Routine plasmid DNA isolation such as for screening for recombinants was done following the alkaline lysis method, while Plasmid DNA for use in cloning and sequencing experiments was done using the NucleoBond[®] PC 100 Midiprep kit (Macherey-Nagel, Germany).

3.1.8.1. Alkaline lysis plasmid DNA isolation

Isolation of plasmid DNA was done using alkaline lysis (mini prep) method. A colony from the transformed plated was inoculated in a test tube with 2 mL of LB medium and ampicillin. The tubes were kept in a shaker incubator at 37 °C overnight. The culture was pipetted into a 1.5 mL micro centrifuge tube and centrifuged at 13000 g for 30 seconds. The supernatant was decanted into a beaker. The bacterial cell pellet was suspended in 100 μ L of ice cold alkaline lysis solution I by vortexing vigorously. Two hundred micro litres of alkaline lysis solution II was measured into the tube and mixed by inversion. One hundred and fifty micro litres of chilled alkaline lysis solution III was measured into the tube and mixed by inversion. The tube was chilled for 5 minutes and centrifuged at 13000 g for 5 minutes at 4 °C. The supernatant was pipetted into a new tube. The DNA was made to precipitate out of the supernatant by adding 2 volumes of absolute ethanol kept at room temperature. The DNA was pelleted by centrifugation. The supernatant was decanted and placed upside down to allow the fluid drain out. One milli liter of 70% ethanol was added to the tube and centrifuged at 13000 g for 2 minutes at 4 °C. The supernatant was decanted. The tube was left to dry so the ethanol can evaporate. Fifty micro litres of TE buffer was pipetted into the tube to suspend the pellet. The DNA solution was stored at -20 °C until further use. The concentration of the plasmid DNA

was determined using Thermo Fisher Scientific NanoDrop 2000 spectrophotometer. Quality and presence of DNA was visually analysed by agarose electrophoresis.

To verify that there is insert in the clones, restriction enzymes were used to digest the isolated plasmid DNA from white colonies.

3.1.8.2. Restriction Endonuclease Digestion

The digestion reaction containing 1 µg of DNA, REN buffer, sterile distilled water and 1 unit of restriction endonuclease was pipetted into a sterile tube and kept in an incubator at 37 °C for 2 hours. The digestion was brought to a halt when 2 µL of 0.1 M EDTA was pipetted into the tube. The concentration of the DNA was determined using Thermo Scientific NanoDrop 2000 spectrophotometer. The quality of the plasmid DNA and presence of insert was visually analysed by agarose electrophoresis, where ten micro litres of the plasmid was analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

3.1.8.3. Colony PCR

Colony PCR was carried out in a Bio-Rad T100 Thermal Cycler. It was done in a 25 µL reaction volume containing sterile distilled water, 2 mM MgCl₂, 1 x Taq Ampliqon II Master Mix, 0.2 µM VsDef P5 F1 primer, 0.2 µM VsDef P5 R1 primer and diluted recombinant bacteria cells. The amplification initiated with a preliminary denaturation step at 95 °C for 5 minutes, followed by 34 cycles of 95 °C for 30 seconds, 49.1 °C (VUG) for 30 seconds and 72 °C for 30 seconds. The reaction was concluded with extension at 72 °C for 5 min, followed by storage at 4 °C. The quality of the plasmid DNA was visually analysed by agarose electrophoresis, where ten micro litres of the plasmid was analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour. Once the clones were verified to be recombinant, they were sent for sequencing at Inqaba Biotec™ and the data obtained was analysed.

3.1.9. Reverse Transcriptase Polymerase Chain Reaction (RT - PCR)

Reverse Transcriptase PCR was done in a Bio-Rad T100 Thermal Cycler. It was done in a 50 µL reaction.

3.1.9.1. One Step Reverse Transcriptase PCR (One – Step RT – PCR)

One Step Reverse Transcriptase PCR was done using OneTaq® One-Step RT-PCR Kit (New England Biolabs, England) in a reaction volume containing sterile distilled water, 1 x OneTaq® Reaction Mix, 0.4 µM reverse primer (Oligo dT), 0.4 µM forward primer (VIG F1), 1 x OneTaq®

Enzyme Mix and 130 ng of *V. subterranea* template RNA sample. The amplification processes started with reverse transcription step at 48 °C for 1 minute, initial denaturation at 94 °C for 1 minute, 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 51.3 °C for 30 seconds and final extension at 68 °C for 1 minute. The reaction was concluded with a final extension at 68 °C for 5 min, followed by cooling at 4 °C prior to storage at -80 °C. The quality of the amplicons was visually analysed by agarose electrophoresis, where ten micro litres of the RT-PCR amplicons were analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

3.1.9.2. Moloney Murine Leukemia Virus (MMLV) RT – PCR

Reverse Transcriptase PCR was done to make a single stand of cDNA. It was done using Invitrogen™ IBW MMLV RT – PCR kit (Thermo Fisher Scientific, South Africa) in a reaction volume containing 0.4 µM reverse primer (Oligo dT), 130 ng of *V. subterranea* template RNA sample and sterile distilled water to make volume 50 µL. The mixture was denatured by incubating at 65 °C for 5 minutes then chilled on ice. 4 µM DTT, 400 U MMLV – RT enzyme and 1x IBW MMLV Buffer was pipetted into the tube which was stored in an incubator at 37 °C for 1 hour, followed by incubation at 70 °C for 10 minutes. Then, the tube was stored at -80 °C until further analysis.

3.1.10. Sequence Analysis and In Silico Characterization

Chromatograms were analyzed and the first 20 bases were removed due to background noise. The chromatograms were edited with Chromas® to replace unclear nucleotide bases with corresponding bases in IUPAC nucleotide codes based on the peaks. The sequences were trimmed with Bio Edit® to remove the M13 coding region of the plasmid. DNA MAN® was used to translate the sequence into proteins of 6 frames. The nucleotide sequences were analysed on the NCBI database using BLASTN search. [ORF finder](#) was used to analyse the deduced amino acid sequence of the defensin gene. Analysis of the amino acid sequence was done on NCBI website using [protein BLAST](#). ClustalW was used to align the amino acid sequences of some defensins from plant.

Homology modeling

The 3D structure of *VsDef1* was created based on models on SWISS-MODEL server (<https://swissmodel.expasy.org>). The template with atleast 40% similarity to sequence of the target protein was used for build the models to determine the structure of *VsDef1* defensin gene.

3.1.11. *In vivo* gene expression profiling

Total RNA was obtained from germinating *V. subterranea* using RNeasy Plant Mini Kit (Qiagen, United States). The RNA concentration was measured with Thermo Scientific NanoDrop 2000 spectrophotometer. The quality and presence of RNA were determined by agarose electrophoresis (section 3.1.11.1). Five micro litres of the samples were analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour. Genomic library construction was done by following the manufacturer's protocol (Lucigen, 2018). Sequencing analysis was done to analyse the DNA nucleotide sequences. Amino acid sequence alignment was also done.

3.1.11.1. RNA Extraction

Total RNA from *V. subterranea* was obtained with RNeasy Plant Mini Kit following the protocol by the manufacturer (Qiagen, United States). Half a gram of embryo and cotyledon of *V. subterranea* at different germinating stage (Day 0, 3 and 6) was weigh into respective tubes and ground with a micro pestle. Four hundred and fifty micro litres of Buffer RLC was added to the tube. The lysate was pipetted into a QIAshredder spin column positioned in a sterile collection tube and centrifuged at 11 000 rpm for 2 minutes. Supernatant was pipetted into a new micro centrifuge tube. Two hundred µL of 100% ethanol was pipetted into the tube and content was mixed with the pipette tip. Then, the sample was transferred into an RNeasy spin column placed in a collection tube and centrifuged at 11 000 rpm for 15 seconds and the flow through was thrown out. Seven hundred µL of RW1 Buffer was pipetted into the column and centrifuged at 11 000 rpm for 15 seconds. The flow through was once again thrown out. Five hundred µL of RPE Buffer was pipetted into the column and centrifuged at 11 000 rpm for 15 seconds and the flow through thrown out after centrifugation. Five hundred µL of RPE Buffer was pipetted into the column and centrifuged at 11 000 rpm for 2 minutes, after which the flow through was thrown out. The column was centrifuged at 11 000 rpm for 1 minute as a dry spin and then cautiously placed in an RNase free tube. Thirty micro liters of sterile water was pipetted onto the column matrix and it was centrifuged for 1 minute. The eluted RNA were treated with Invitrogen™ DNase (Thermo Fisher Scientific, South Africa) and stored at -80°C until use. The concentration of RNA was determined using Thermo Scientific NanoDrop 2000 spectrophotometer. To determine the quality of the RNA, 5 µL of the samples were analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

3.1.11.2. Reverse Transcription Real Time PCR (RT-qPCR)

The RT-qPCR reactions were set up for relative and absolute quantification with Kapa SyBr PCR kit (Kapa Biosystems, South Africa) according to manufacturer's recommendations in a Bio-Rad CFX96 Real-time System (Bio-Rad). Gene-specific primers (VsDef P1) were used for RT-qPCR assay. Both absolute and relative quantification was done to measure the expression level of defensin mRNA in *Vigna subterranea*. The house-keeping β -tubulin gene was evaluated as an internal reference gene. Absolute quantification was done with serially diluted standards with a known initial RNA concentration to create a standard curve which determines the concentration of unknown samples according to the Ct values. Assay was done in a 20 μ L reaction volume containing sterile distilled water, 1 x Kapa SyBr Enzyme Mix, 0.2 μ M VsDef P1 R1 primer, 0.2 μ M VsDef P1 F1 primer, 0.2 μ M ROX high reference dye, 1 x Kapa SyBr RT Mix, diluted *V. subterranea* template RNA sample and sterile distilled water to top up volume to 20 μ L. In order to ensure uniform distribution of reagent and minimize pipetting errors, two master mixes were made separately without adding RNA. Reference primer (β -tubulin) was added to one master mix while the other master mix was for VsDef P1 primers. Additional two μ L water was added into water control (WC) tubes for both the reference gene (β -tubulin) and experimental reaction. A No Reverse Transcriptase Control was also added in the setup for the reaction. The following cycle protocol was set up as follows: enzyme activation at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 3 seconds, annealing at 51.3 °C, extension at 72 °C and data acquisition for 20 seconds. The reaction was concluded with extension at 60 °C for 5 min. The tubes were stored at 4 °C. The results were analysed using Bio-Rad CFX96 Maestro software.

3.1.12. Determining Full Sequence of Recombinant Clones

Labelling and Detection of specific colonies of interest were done according to manufacturers' protocol using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, South Africa). Genomic Library Construction was done according to Maniatis Molecular Cloning Protocol (Sambrook *et al.*, 1989).

3.1.12.1. Genomic DNA Fragmentation

Total *V. subterranea* DNA (7 x 5 μ L) was pipetted into 1.5 mL micro centrifuge tubes. The concentration of the DNA was diluted with 5 μ L Tris – EDTA (TE) buffer added to each tube. Four methods (vortex for 1 min and 5 minutes; pipetting with P200 5 times and 30 times; using sonicator for 1 minute and 5 minutes; using syringe for 1 min) were used in order to determine the best way to fragment the DNA. Ten μ L of the fragmented DNA was visually analysed on a

1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1 x TAE) at 100 Volts for an hour.

Five µL of total *V. subterranea* DNA (137.7 ng/µL) was pipetted into a sterile micro centrifuge tube. The concentration of the DNA was diluted with TE buffer to 68 ng/µL. The DNA was pipetted 40 times to fragment it. One hundred and thirty µL of TE was pipetted into the tube to make the volume 140 µL. One hundred µL of the DNA was stored in the freezer at -20°C. The remaining 40 µL of the DNA was ran on a 0.5% agarose gel using electrophoresis buffer (1x TAE) at 40V for 3 hours. Half of the gel containing the molecular weight marker, unfragmented DNA (control) and fragmented DNA was stained with buffer solution with SYBR Green for 30 minutes. Transparent paper was placed on the gel while viewing under UV light to mark the position of the fragmented DNA. The bands containing the rest of the fragmented DNA was cut out from the other half of the gel using the transparent paper as a guide. Clean up of that DNA was done using Nucleospin® Gel method according to the manufacturer's recommendations (Macherey-Nagel, 2017).

3.1.13. End – Repair of Genomic DNA Fragments

End – Repair of DNA fragments was done using a Lucigen's End-It™ DNA End-Repair Kit according to protocol provided by the manufacturer (Lucigen, 2016). It was done in a 25 µL reaction volume made up of 15 µL fragmented DNA (68 ng/µL), 2.5 µL 10x DNA Terminator End Repair Buffer, 1 µL DNA Terminator End Repair Enzymes and 6.5 µL sterile water. The tube was stored at room temperature for half an hour. Followed by incubation at 70°C for 15 minutes to halt the reaction

Purification of repaired DNA fragments was done using the phenol chloroform method. Two hundred micro liters of phenol stabilised in TE solution was added to the tube. After inverting the tube, it was centrifuged at 13000 rpm for 5 minutes. The top layer was pipetted into a sterile 1.5 mL micro centrifuge tube. Two hundred micro liters of phenol solution mixed with chloroform was pipetted into the tube which was centrifuged at 13000 rpm for 5 minutes and the top layer was pipetted into a new sterile tube. Two hundred micro liters of chloroform was then pipetted into the tube which was centrifuged at 13000 rpm for 5 minutes. The top layer was poured out and the pellet was left to dry for 15 minutes. The pellet was dissolved in 30 µL of RNA-free water and the purified DNA was stored at -20°C.

3.1.14. Ligation to the pJAZZ® Vector

Purified fragmented DNA was ligated to pJAZZ® Vector by preparing a 10 µL reaction volume containing blunt ends fragmented DNA (68 ng/µL), 0.5 x pJAZZ-OC Vector, 1 x CloneDirect Ligation buffer and 2 units of CloneSmart DNA Ligase. The tube was placed in an incubator

at 25 °C for 2 hours, followed by a 15 minute incubation at 70 °C, and lastly a cooling step at room temperature for 15 seconds. The tube was then stored at 4 °C until further analysis.

3.1.15. Transformation of BigEasy TSA™ Electrocompetent Cells

BigEasy TSA™ cells were allowed to thaw completely on ice. One µL of heat treated ligation reaction was added to 25 µL cells. The mixture was gently stirred with a pipette tip. Twenty five µL of the cells/DNA mixture was pipetted into an electroporation cuvette chilled on ice. The cuvette was positioned in the Eppendorf Electroporator 2510 and pulsed for a few seconds at 1800 Volts. Nine hundred and seventy five micro liters of SOC medium was pipetted into the cuvette to replenish and suspend the cells. The cell mixture was pipetted into sterile 1.5 mL micro centrifuge tubes and incubated at 37 °C for 1 hour in a shaking incubator (250 rpm). The transformed cells were spread on 2YT plates made with chloramphenicol, IPTG and X-gal. The plates were incubated at 37 °C for 16 hours.

3.1.16. Evaluation of Genomic Library

Ten white colonies were subcultured from different plates and inoculated in 10 x 5 mL of 2YT broth containing chloramphenicol. The tubes were incubated overnight at 37 °C. An alkaline lysis mini prep to isolate the Plasmid DNA from the cells was done. Ten micro liters of the plasmid DNA was analysed on a 1% agarose gel stained with Pronasafe™, using electrophoresis buffer (1x TAE) at 100 Volts for an hour.

3.1.17. Binding of DNA of Lysed Cells to Filters

Sterile dry nitrocellulose filter (8 mm) was labelled with a soft-lead pencil. One filter was placed on each of the 2YT plates containing the transformed cells of genomic library. The plates with the filters were incubated at 37 °C for 6 hours. The filter was peeled from the plate using a blunt-ended forceps. The filter was then placed onto new agar plates to make a second replica plate. The plates (replica and master) were stored in an incubator at 37 °C to regrow the colonies for 4 hours. The plates were parafilm-wrapped and stored at 4 °C in an inverted position. Whatman 3 MM paper (4 x 8 mm) was placed in plastic tray and saturated with solutions 1 to 4 respectively: 10% SDS solution, denaturing solution, neutralizing solution and 2X SSC solution. The filter was moved onto a 3 MM paper flooded with 10% SDS solution and left on it for 1 for 3 minutes. The filter was then moved to next paper soaked with denaturing solution to allow it get exposed to the solution for 5 minutes. The filter was moved to the next paper flooded with neutralising solution for 5 minutes. The filter was then finally transferred to the last paper soaked with 2 x SSC solution for 5 minutes. All the filters were processed in the same order. The filters were left to dry on a paper for half an hour. The DNA

on the filter was cross-linked with UV light by exposing the filters to UV for 2 minutes. The filters were stored at 4 °C until further use.

3.1.18. Labelling of Probe for Genomic Library

One µg of PCR amplicon (cloned DNA fragment) was pipetted into a sterile tube and 14.5 µL of sterile water was pipetted into the tube. It was boiled for 10 minutes to denature the DNA, followed by immediately chilling on ice. Four µL of DIG-High Prime was pipetted into the tube which was then stored overnight at 37 °C in a shaking incubator. 2 µL of 0.2 M EDTA was pipetted into the tube to halt the reaction. The tube was kept at -20 °C until further use.

3.1.19. Screening of Bacterial Colonies by Hybridization

Forty mL of DIG Easy Hub Buffer was preheated to hybridisation temperature of 45 °C. Five mL of the buffer / 50 cm² filter was added to a closed Ziploc bag containing the filters and it was incubated with gentle agitation for 30 minutes in a hybridisation oven at 45 °C. The pre-hybridisation solution was poured out of the bag. The tube containing DIG-labelled probe for DNA was boiled for 5 minutes and then chilled. The denatured probe was pipetted into 15 mL of the preheated DIG Easy Hub Buffer. The probe/hybridisation buffer was added into the bag and stored with gentle agitation in a hybridization oven at 45 °C for 16 hours. Afterwards, the DIG Easy Hub Buffer containing the probe was poured out of the Ziploc bag into a container and stored at -20 °C. The first stringency wash was done by adding about 30 mL of wash solution (containing 2 x SSC and 0.1% SDS) and keeping the bag at room temperature with gentle agitation for 5 minutes. The wash solution was then poured out. The 2nd stringency wash was done by adding about 30 mL of wash solution (containing 0.5 x SSC and 0.1% SDS) and incubating the bag for 5 minutes at 65 °C. The wash solution was poured out.

3.1.20. Non-Radioactive Immunological Detection

The filters were washed with washing buffer (containing 0.3% v/v Tween 20, 0.15 M NaCl and 0.1 M Maleic acid). One hundred mL of 1 x blocking solution was added to a large container containing the filters and incubated with gentle agitation for 30 minutes at 25 °C. The blocking solution was discarded. Fifty milli liters of antibody solution (containing 75 mU/mL Anti-digoxigenin-AP) was added to a container and incubated with gentle agitation for 25 minutes at 25 °C, after which the solution was discarded. 2 x 5 minutes wash was done in 100 mL of washing buffer. Fifty milli liters of 1 x detection buffer was measured into the container and incubated with gentle agitation for 10 minutes at 25 °C. The solution was discarded. The enzymatic reaction was done by incubating the membrane in 50 mL of new substrate solution (containing 1 mL of 50 x BCIP/NBT solution and 49 mL of detection buffer) at room temperature in the dark, overnight. Rinsing the filters with sterile distilled water for a few seconds was done

to halt the reaction. The developed membrane filters were allowed to air-dry to document the results.

3.1.21. Determination of Copy Number of Gene

Southern blotting was done to know how the defensin genes are organised in genomes by mapping restriction sites in the DNA fragments. *V. subterranea* genomic DNA (3 x 4000 ng) was cut with enzymes (*Hind III*, *Bam HI*, and *Bam HI/ Hind III* respectively) overnight at 37 °C. The digested DNA was ran on a 0.8% agarose gel stained with Pronasafe™, using buffer (1x TAE) at 20V for over 8 hours. The gel was photographed under UV light. The gel portion with no DNA was removed with a scalpel. The gel was transferred into a clean dish. Twenty mL of denaturing solution (0.5 M NaOH, 1.5 M NaCl) was measured into the dish and kept at 25 °C with gentle agitation for 30 minutes. The gel was rinsed briefly in distilled water. Twenty mL of neutralising buffer (1M Tris, 1.5 M NaCl) was measured into the dish and kept at 25 °C with gentle agitation for 30 minutes. The neutralising buffer was changed and an additional 15 minutes incubation was done, after which the buffer was discarded. Nitrocellulose membranes and two Whatman paper were cut to fit the gel. The membrane was immersed in water, then immersed in transfer buffer (10 x SSC). One blotting paper (wick) was placed on a tray with its ends draping into the transfer buffer in a container. The gel was flipped and centred on the wet blotting paper. The wet nitrocellulose membrane was positioned on top of the gel followed by the wet paper. Air bubbles were removed by rolling a pipette on the paper, after which paper towels was added. A plate was placed on the top and pressed down with a weight. The DNA was allowed to transfer for about a day. The papers were removed from the gel. The gel and the membrane was turned over and laid on a new blotting paper and the position of the gel slots were marked on the membrane. The gel was peeled off and discarded. The membrane was soaked in 6 x SSC buffer and stored at 25 °C for 5 minutes. The DNA was fixed by baking the membrane in a microwave oven for 3 minutes. The immobilized DNA was hybridised with appropriate probe.

CHAPTER FOUR

4.1. Results

The inhibition of bacterial growth on plates was noticed during inhibition zone assays with *Vigna subterranea* seeds and embryos. Therefore, to isolate the particular genes responsible and characterize them, high molecular weight DNA was successfully isolated from *V. subterranea* leaf tissues. The result is shown in Figure 4 below.

4.1.1. DNA Isolation

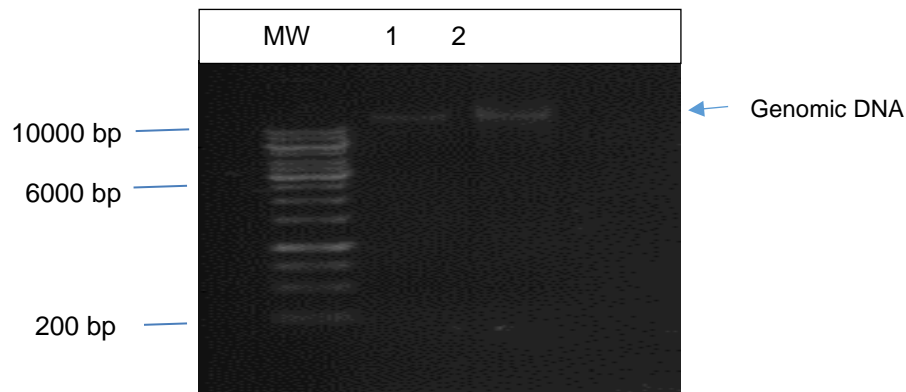


Figure 4: Photograph of a Pronasafe™ stained agarose gel showing size of total DNA isolated from *Vignasubterranea*. The lanes represent: MW – λ -PstI digest Molecular weight marker (Fermentas), 1 – *Vigna subterranean* DNA, 2 – *Vigna subterranean* DNA.

Thereafter, the *Vigna subterranea* DNA served as a template for amplification of antimicrobial gene fragments as well as for Southern analysis.

4.1.2. Primer Design

Output of bioinformatics analysis was 4 primers which were synthesized. The results of the bio-informatics analysis are shown in Appendix A, with highlighted primers on the multiple defensins sequences aligned. The primers were used in PCR, RT - PCR and cloning.

4.1.3. Polymerase Chain Reaction (PCR) and Screening for Recombinants

To clone and analyse these genes, PCR was done to determine optimum annealing temperature and magnesium chloride. The results are shown in figure 5 to 11. In each case, the figures show successful amplification as well as temperature and/or magnesium chloride optimization of fragments of expected sizes, as well as result of screening for recombinants and BLAST analysis.

VsDef P1

VsDef P1 is a primer that was designed to bind to the core region of defensin genes from *Cicer arietinum*, *Medicago truncatula*, *Trigonella foenum-graecum*, *Medicago sativa* and *Datura stramonium*. High molecular weight DNA was successfully used for PCR with VsDef P1 primers designed to amplify a core fragment of *V. subterranea* antimicrobial peptide as shown in the figures below.

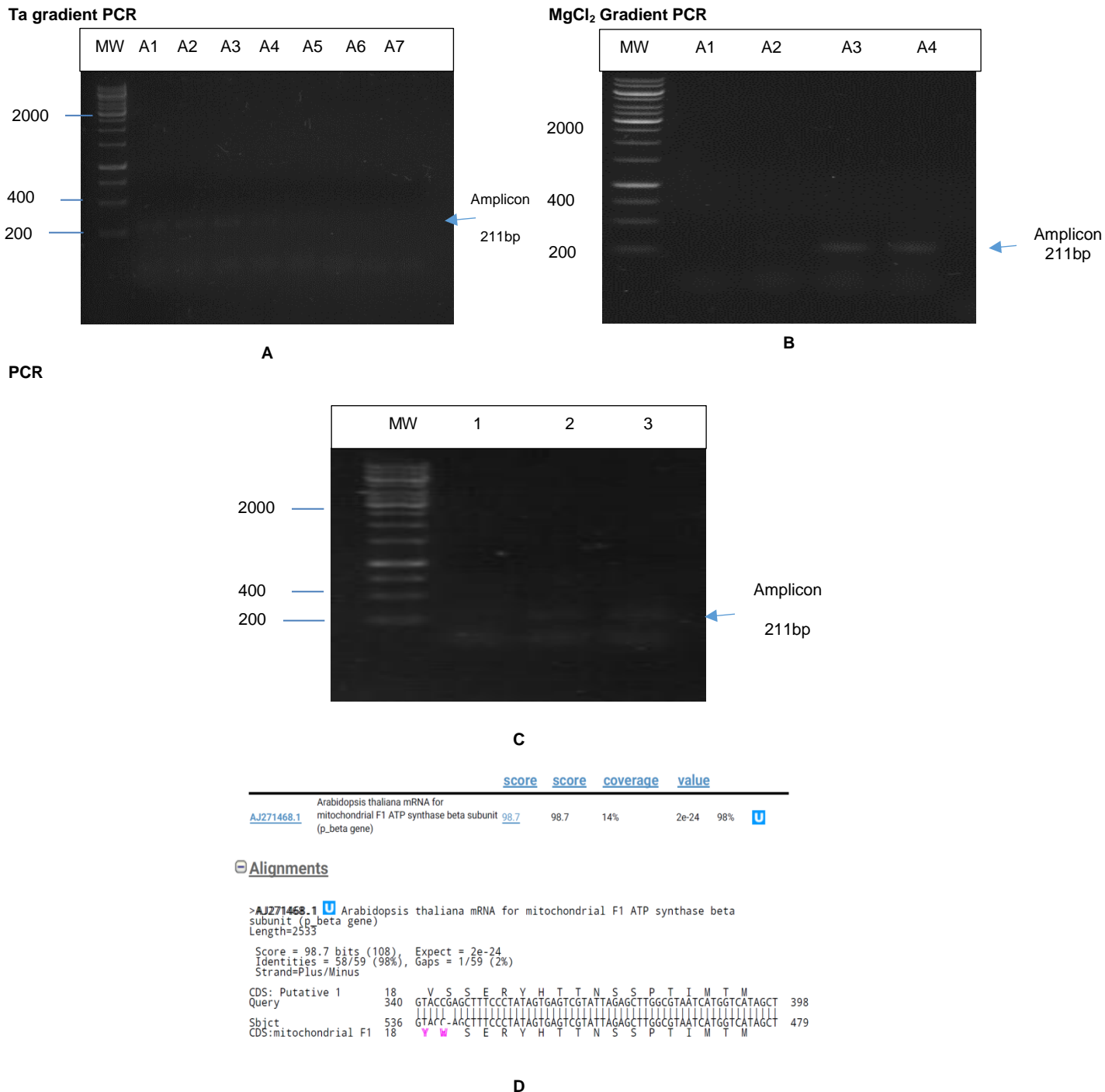


Figure 5: Photograph of a Pronasafe™ stained agarose gels showing size of PCR products (amplicon). **A**, **B** and **C** show successful amplification of a 211 bp fragment of the VsDef P1 gene when *Vigna subterranea* DNA was amplified with VsDef P1 primers at optimum annealing temperature of 47.2°C and MgCl₂ concentration of 3 mM. **D** shows VsDef P1 nucleotide and their aa translation pairwise alignment with *Arabidopsis thaliana* ATP synthase which was the closest match as revealed by BLAST analysis.

VsDef P2

VsDef P2 is a primer that was designed to bind to the core region of defensin genes from *Cicer arietinum*, *Medicago truncatula*, *Trigonella foenum-graecum*, *Medicago sativa* and *Datura stramonium*. High molecular weight DNA was successfully used for PCR with VsDef P2 primers designed to amplify a core fragment of *V. subterranea* antimicrobial peptide as shown in the figures below.

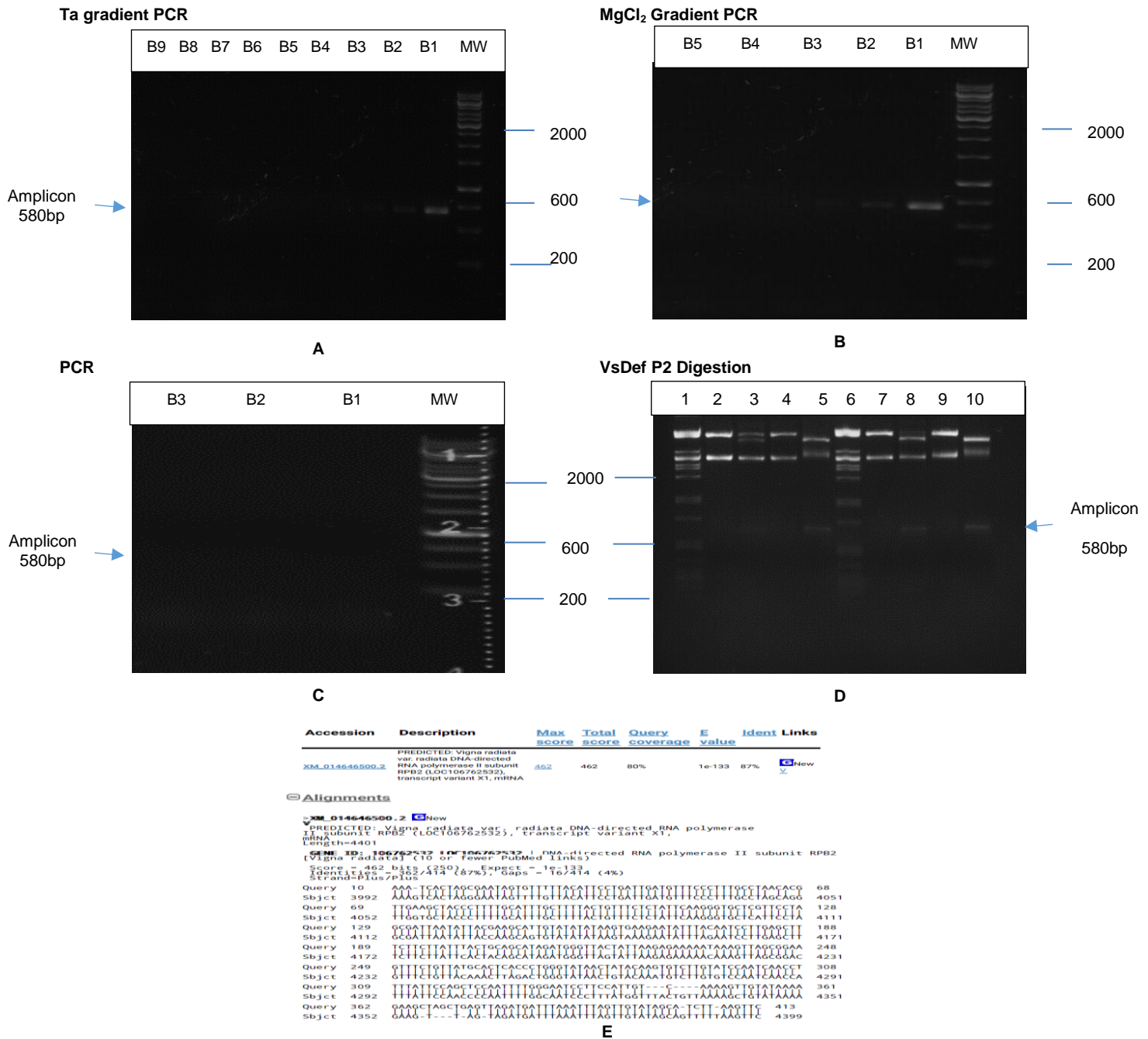


Figure 6: Photograph of a Pronasafe™ stained agarose gel showing size of PCR products. **A**, **B** and **C** show successful amplification of a 580 bp fragment of the VsDef P2 gene when *Vigna subterranea* DNA was amplified with VsDef P2 primers at optimum annealing temperature of 40°C and MgCl₂ concentration of 1 mM. **D** shows the amplicon excised from clones while screening for recombinants while **E** shows VsDef P2 nucleotide and their aa translation pairwise alignment with *Vigna radiata* RNA polymerase which was the closest match as revealed by BLAST analysis.

VsDef P3

VsDef P3 is a primer that was designed to bind to the core region of defensin genes from *Arachis diogoi*, *Trigonella foenum-graceum*, *Cicer arietinum*, *Vicia faba* and *Pisum sativum*. High molecular weight DNA was successfully used for PCR with VsDef P3 primers designed to amplify a core fragment of *V. subterranea* antimicrobial peptide as shown in the figures below.

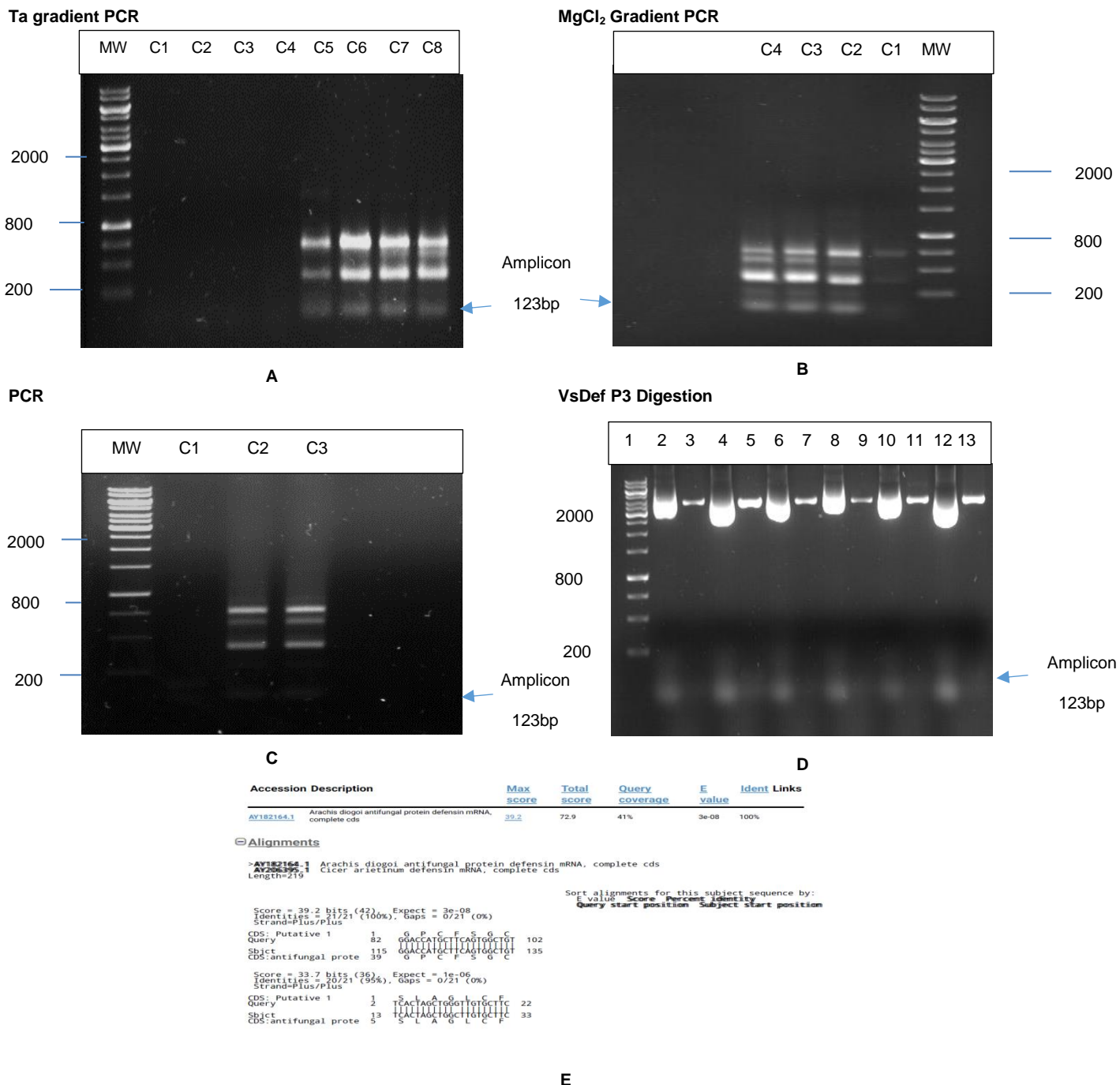


Figure 7: Photograph of a Pronasafe™ stained agarose gel showing size of PCR products. **A**, **B** and **C** show successful amplification of a 123 bp fragment of the VsDef P3 gene when *Vigna subterranea* DNA was amplified with VsDef P3 primers at optimum annealing temperature of 47.2°C and MgCl₂ concentration of 1 mM. **D** shows the amplicon excised from clones while screening for recombinants while **E** shows VsDef P3 nucleotide and their aa translation pairwise alignment with *Arachis diogoi* defensin which was the closest match as revealed by BLAST analysis

VsDef P4

VsDef P4 is a primer that was designed to bind to the core region of defensin genes from *Vigna unguiculata*, *Vigna angularis* var. *angularis*, *Phaseolus vulgaris*, *Vigna radiata* var. *radiata* and *Cajanus cajan*. High molecular weight DNA was successfully used for PCR with VsDef P4 primers designed to amplify a core fragment of *V. subterranea* antimicrobial peptide as shown in the figures below.

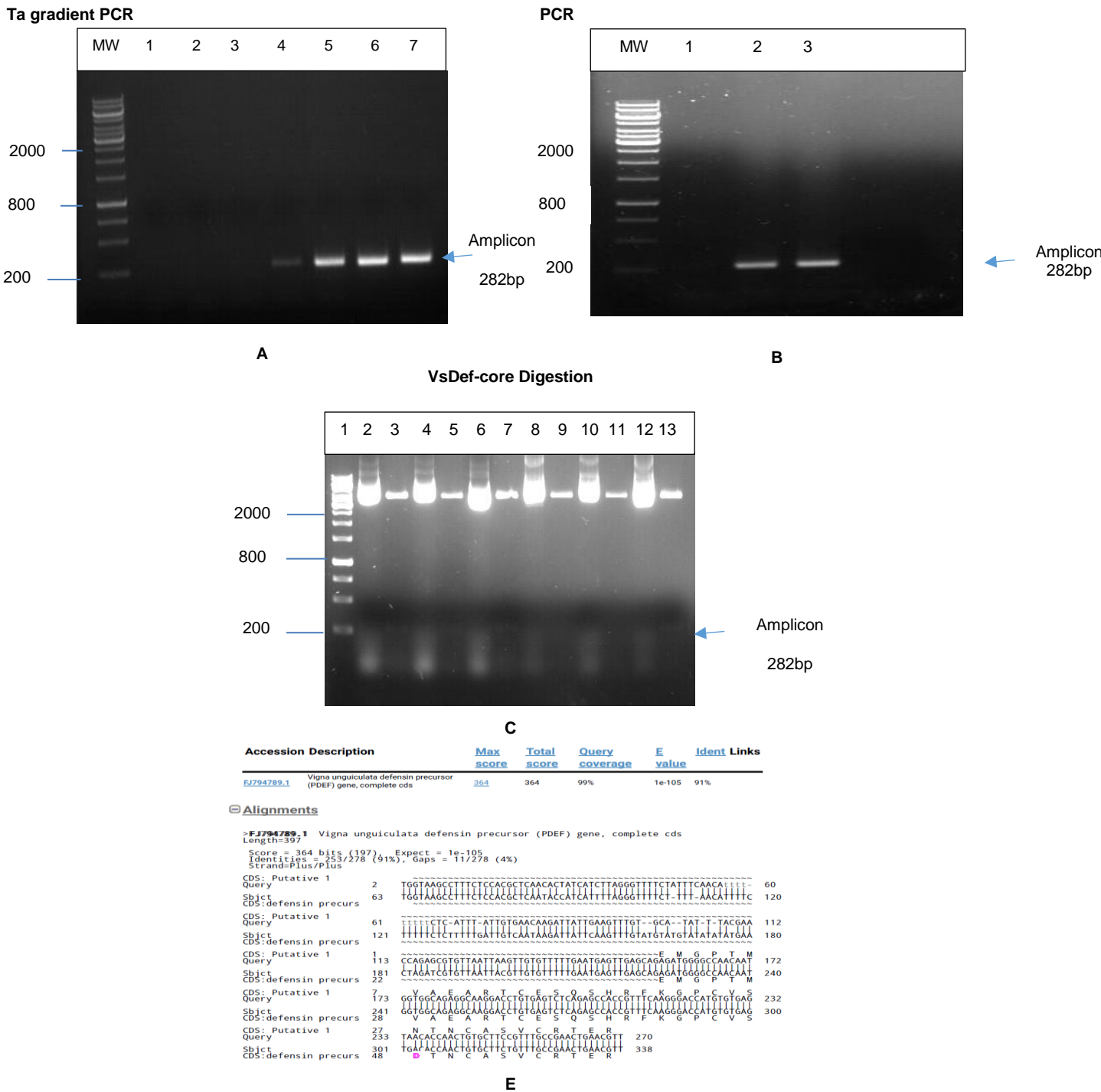


Figure 8: Photograph of a Pronasafe™ stained agarose gel showing size of PCR products. **A** and **B** show successful amplification of a 282 bp fragment of the VsDef-core gene when *Vigna subterranea* DNA was amplified with VsDef P4 primers at optimum annealing temperature of 51.3°C. **C** shows the amplicon excised from clones while screening for recombinants and **D** shows VsDef-core nucleotide and their aa translation pairwise alignment with *Vigna unguiculata* defensin precursor which was the closest match as revealed by BLAST analysis

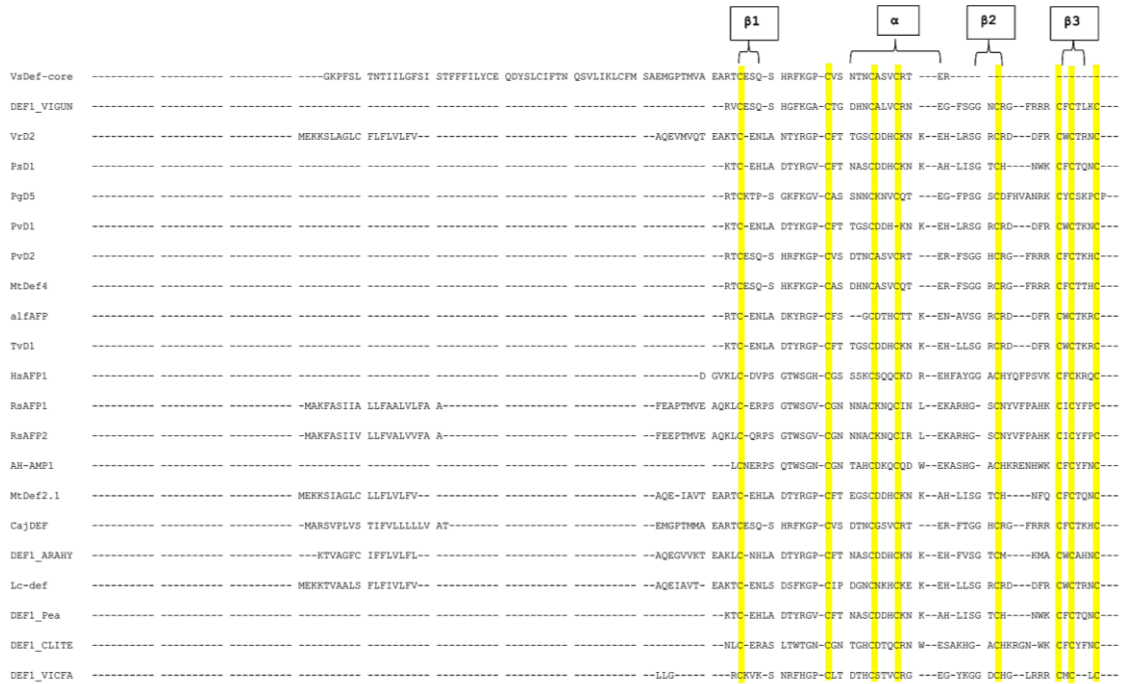


Figure 9: Amino acid sequence alignment indicating conserved CSαβ motif in plant defensins.

The yellow shows the 8 residues of cysteine. The alignment was done with ClustalW (Thompson *et al.*, 1994). The sequences are *VsDef1* from *Vigna subterranea*, DEF1_VIGUN from *Vigna unguiculata*, VrD2 from *Vigna radiata*, PsD1 and Def1_Pea from *Pisum sativum*, PgD5 from , PvD1 and PvD2 from *Phaseolus vulgaris*, MtDef2.1 and MtDef4 from *Medicago trunculata*, alfAFP from *Medicago sativa*, TvD1 from *Tephrosia villosa*, HsAFP from *Heuchera sanguinea*, RsAFP1 and RsAFP2 from *Raphanus sativum*, AH-AMP1 from *Aesculus hippocastanum*, CajDef from *Cajanus cajan*, DEF_ARAHY from *Arachis hypogea*, Lc-Def from *Lens culinaris*, DEF1_CLITE from *Clitorea ternatea*, DEF1_VICFA from *Vicia faba*.

As shown in figure 9 above, the recombinant gene, *VsDef-core* is an incomplete gene. However, the level of sequence similarity between *VsDef-core* and DEF1_VIGUN is very high, so we assumed that primers designed against the *Vigna unguiculata* sequences would amplify the corresponding gene in *Vigna subterranea* which is *VsDef1*.

VsDef P5

When the nucleotide sequence of *Vigna unguiculata* was analysed, it was shown to be similar to that of *Vigna subterranea*, so new primers, VsDef P5 were designed to target the full defensin gene. High molecular weight DNA was successfully used for PCR and RT PCR with VsDef P5 primers designed to amplify the full length of *V. subterranea* antimicrobial peptide as shown in the figures below.

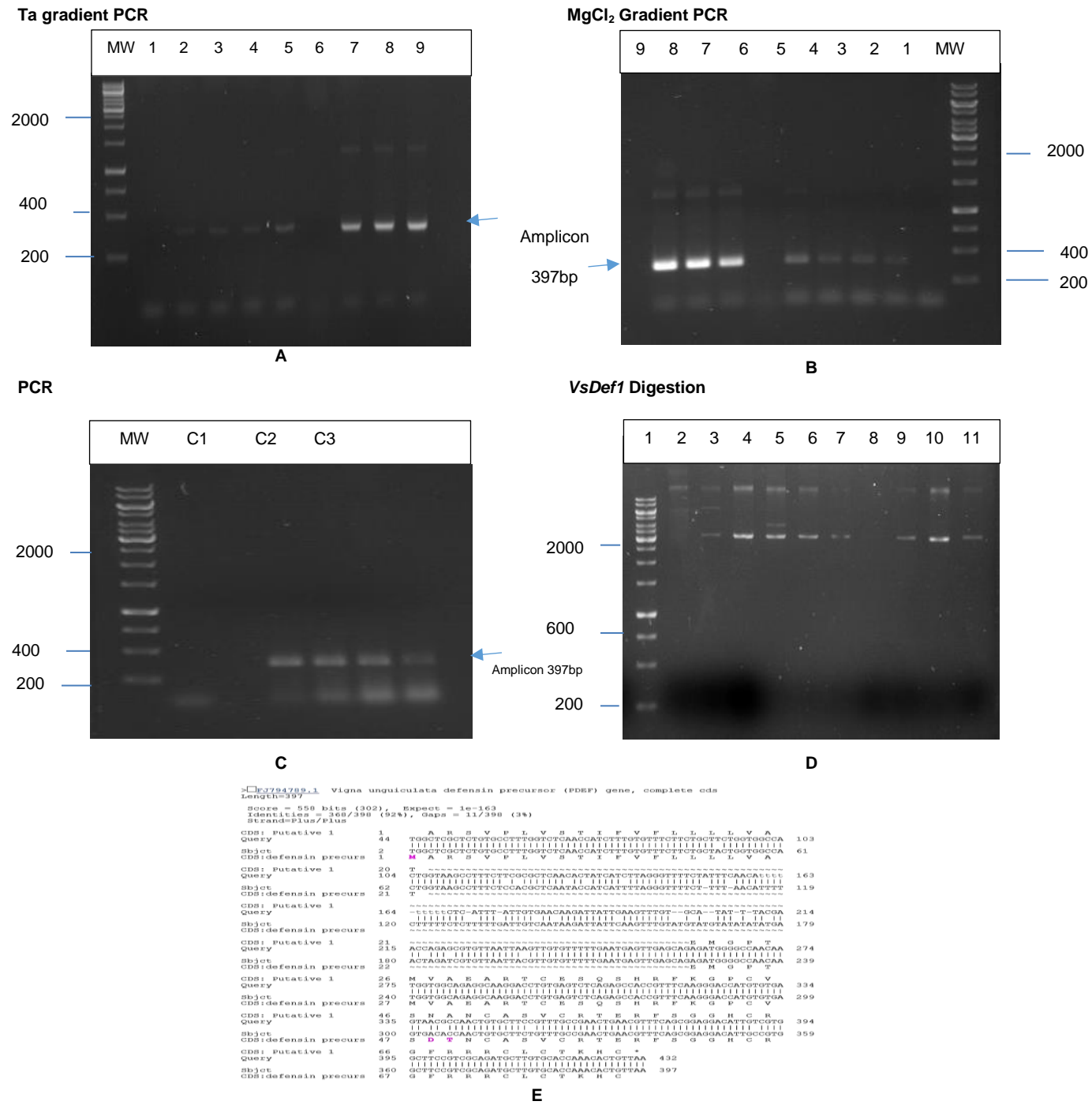


Figure 10: Photograph of a Pronasafe™ stained agarose gel showing size of PCR products. **A, B** and **C** show successful amplification of a 397 bp fragment of the *VsDef1* gene when *Vigna subterranea* DNA was amplified with *VsDef* P5 primers at optimum annealing temperature of 49.1°C and MgCl₂ concentration of 5 mM. **D** shows the amplicon excised from clones while screening for recombinants while **E** shows *VsDef1* nucleotide and their aa translation pairwise alignment with *Vigna unguiculata* defensin precursor which was the closest match as revealed by BLAST analysis

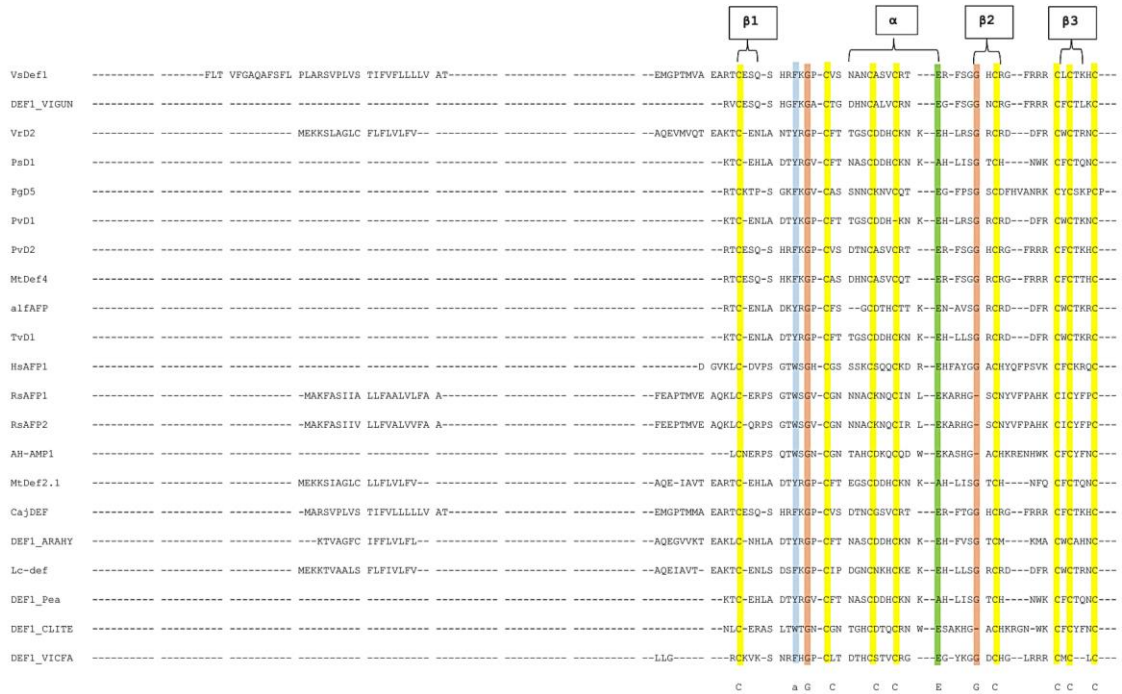


Figure 11: Amino acid sequence alignment indicating conserved CSαβ motif in *VsDef1* as well as other plant defensins.

The consensus sequence for a mature defensin peptide is also included: the cysteine residues are in yellow, aromatic residues in blue, glycine in pink, glutamate in green. The alignment was done with ClustalW (Thompson *et al.*, 1994). The plants used are listed in figure 9.



Figure 12: showing 3D model of *VsDEF1*, modelled with SWISS-MODEL

4.1.4. Genomic Library Construction

After confirming the identity of the gene fragment, genomic library of *Vigna subterranea* was constructed with fragmented high MW genomic DNA so as to determine the full length of the gene.

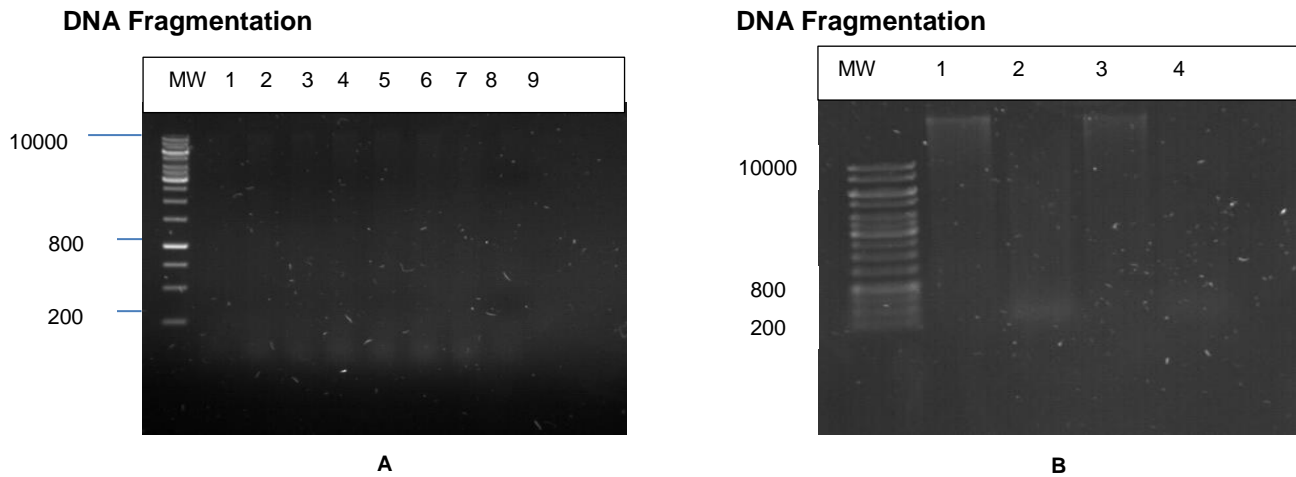


Figure 13: Photograph of a Pronasafe™ stained agarose gel showing smear of fragmented *Vigna subterranea* genomic DNA for library construction

Plasmid DNA for genomic library

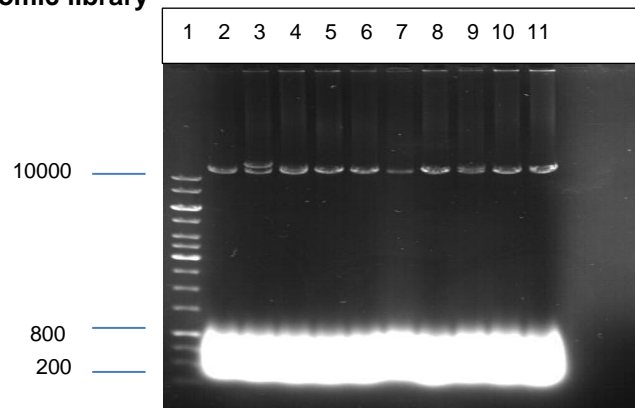


Figure 14: Photograph of a Pronasafe™ stained agarose gel showing size of white clones in pJAZZ vector

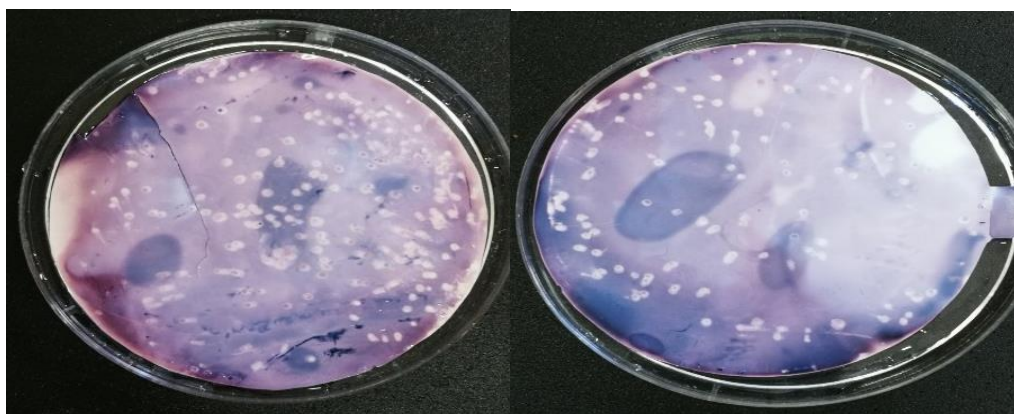


Figure 15: showing non-radioactive immunological detection of hybridized bacterial colonies

No defensin clones were obtained, and further screening was discontinued for two reasons – evidence from the cloning experiment indicated VsDef gene to be highly toxic to *E. coli*, with only mutated or partially deleted clones surviving, and technically, the membranes were old and brittle.

However, *in vivo* characterisation of gene expression patterns was successfully investigated.

4.1.5. Cloning and Characterization of AMPs

	VsDef P1	VsDef P2	VsDef P3	VsDef P4	VsDef P5
Forward primer	VsDef P1 F1	VsDef P2 F1	VsDef P3 F1	VsDef P4 F1	VsDef P5 F1
Reverse primer	VsDef P1 R1	VsDef P2 R1	VsDef P3 R1	VsDef P4 R1	VsDef P5 R1
Size of amplicon: obtained	211 bp	580 bp	123 bp	282 bp	397 bp
Clonable in <i>E. coli</i>	yes	yes	yes	yes	no
BLAST analysis	DEF 3' fragment	RNA polymerase	<i>Arachis</i> and <i>Cicer</i> defensin fragment	VsDef-core internal fragment	<i>VsDef1</i> precursor gene

4.1.6. RNA Extraction

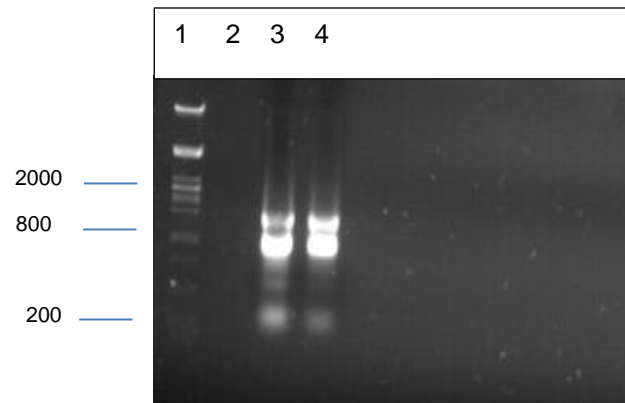


Figure 16: Photograph of a Pronasafe™ stained agarose gel showing size of total RNA isolated from *V.subterranea*. The lanes represent: 1 – λ -PstI digest Molecular weight marker, 2 – Blank, 3 – *Vigna subterranea* RNA, 4 – *Vigna subterranea* RNA.

4.1.7. Reverse Transcriptase RT – PCR

RNA samples were used for RT PCR and Real Time PCR (qualitative and quantitative analysis) in order to determine the expression pattern of defensin by *Vigna subterranea*.

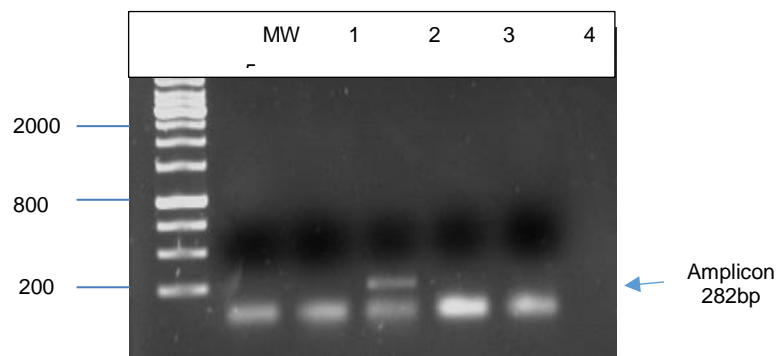


Figure 17: Photograph of a Pronasafe™ stained agarose gel showing size of total RNA isolated from *V. subterranea*.

4.1.8. *In vivo* defensin gene expression profiling

The expression of β -tubulin was used as a reference gene to analyse defensin expression. Relative and absolute quantification was done.

Validation of reference gene

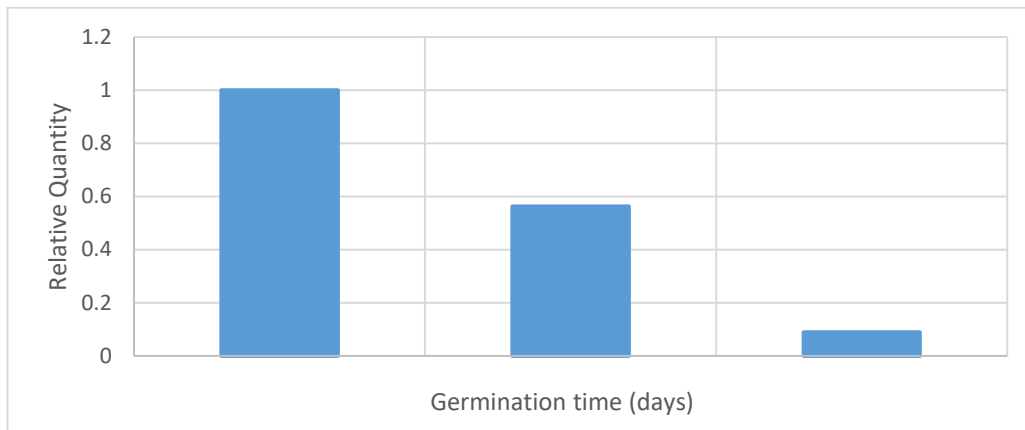


Figure 18: Bar graph showing β -tubulin mRNA expression analysed using RT-qPCR of plants with different germination days shows highest expression in D0 embryo, and β -tubulin mRNA levels decrease by almost 50% by Day 3, and also ten-fold by D6.

Quantification of *VsDef1* mRNA in *Vigna subterranea*

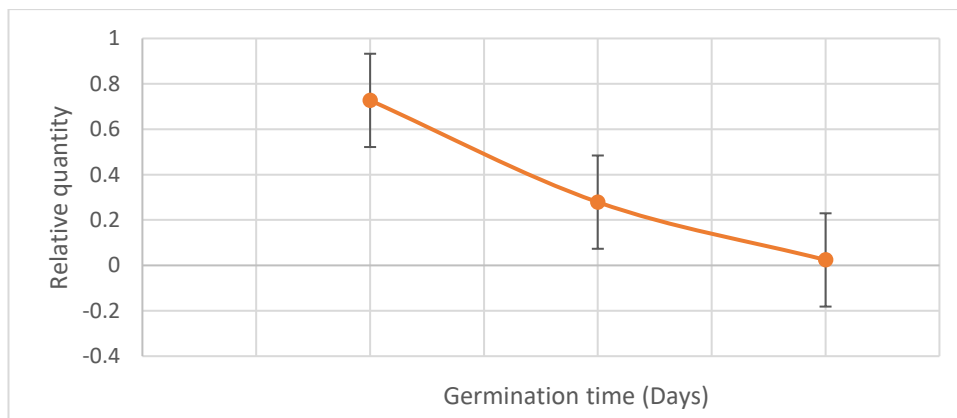


Figure 19: Line graph showing *VsDef1* mRNA expression analysed using RT-qPCR of plants with different germination days

The highest levels of *VsDef1* mRNA were detected in the embryo at Day 0. The levels decrease through Day 3 to very low levels (less than 10% of Day 0 levels) by Day 6.

4.2. Discussion

Results of the zone inhibition experiment demonstrate that antimicrobial compounds / proteins are expressed in developing embryo of *Vigna subterranea*. This is in agreement with Ajiboye and Oyejobi (2017) who previously demonstrated that the extracts from seeds of *Vigna subterranea* have antimicrobial activity against some micro-organisms; the most prominent activity was noticed against *Escherichia coli* (10^{-1} dilution) with 24 ± 2.83 mm zone of inhibition while the most minimal activity was observed against *Pseudomonas aeruginosa* (10^{-1} dilution) with 10 ± 1.87 mm zone of inhibition. However, our results (Figure 19) suggested that the antimicrobial proteins are transcribed and translated during the developmental stages of the embryo.

Decision to use *V. subterranea* total genomic DNA as template is because plants are known to have several homologs of antimicrobial proteins (defensins), both functional and non-functional homologs (Franco, 2011). By doing polymerase chain reaction, we wanted to pick all the copies of the defensin genes that were present in the plant. Therefore, we embarked on a PCR cloning approach to clone as many homologs as possible. However, only one homolog was successfully cloned (Figure 8).

High copy number of defensin genes has been discovered in model plants i.e. *Arabidopsis thaliana* and *Medicago truncatula* that are well studied but in orphan crops, it is difficult to identify these genes because of extreme diversity in the sequences. Since common molecular methods like polymerase chain reaction or hybridization only enable identification of sequences that are closely related. Mergaert *et al.* (2003) identified nineteen out of the forty two *M. truncatula* cDNAs as defensin (DEFL) genes that were induced during nodule development. The diversity and number of these genes expressed by *M. truncatula*'s nodules provided an extensive base to identify defensin genes from other species. Therefore, we need to screen more independent clones of the defensin homologs in *Vigna subterranea* with methods such as fluorescence in situ hybridization (FISH), paralogue ratio test, quantitative RT-PCR.

In an effort to demonstrate the copy number of defensin in *Vigna subterranea*, we created a *V. subterranea* genomic DNA library in BigEasy® v2.0 Linear Cloning System (pJAZZ® Vectors). Southern analysis would give an idea of the copy number of the defensin genes present. Screening of the library by colony hybridization initially identified possible clones (Figure 15). However, further analysis by restriction endonuclease analysis and PCR screening did not confirm any of the clones as positive defensin (*VsDef1*) clones. In future,

the experiment will be repeated in a more tolerant host, such as yeast, though, our laboratory does not currently have the capacity for eukaryotic cloning vectors.

Stotz *et al.* (2009) discovered that uninterrupted expression of some defensins in some transgenes are harmful to the plants even though there are studies available on the successful expression of some functional plant defensins in transgenic plants like *A. thaliana*, tomato and cotton. For example, the adverse effects range from reduction in cell growth and regeneration efficiency, to odd morphology of the regenerated transgenic plants and reduction in plant fertility. These effects vary based on expression level, tissue in which expression occurs and the growth stage of the host plant during the expression (Stotz *et al.*, 2009).

Anderson *et al.* (2009) modified a chimeric defensin which consists of a domain of mature *Nicotiana glauca* defensin, NaD1 fused with a C-terminal propeptide domain (CTPP) so as to reduce the toxic effects of expressed defensins on host plants. Without a CTPP, the mature NaD1 causes short internodes and deformed leaves in host cotton plant. Meanwhile, a NaD1 gene fused with CTPP do not have these effects on host cotton plant, which means CTPP protects the plants or enable the peptide to move to appropriate tissue i.e. vacuole. The result of restriction endonuclease analysis of the putative *VsDef1* clones (Figure 8C) indicate that deletion is more likely the scenario since insert bands were very faint on agarose gel because the toxic gene was being expressed in *Escherichia coli* which might kill the host or gets deleted so the cells can survive.

Amino acid sequence analysis of plant defensins (Figure 11) revealed that the peptides share homology at amino acid level. *VsDef1* was found to be 95% similar to *Vigna unguiculata* defensin. Multiple sequence alignment of *VsDef1* with sequences of leguminous defensins in NCBI database reveals that the fragment encompasses the β 1 - 3 and alpha helix fragment of defensin gene. When defensins from plants are so similar, the variance in the amino acid sequences is crucial to determine their novel antimicrobial activities (Rogozhin *et al.*, 2018; Kaewklom *et al.*, 2018).

Plant defensins have a structure with a stable motif with cysteine (known as CS α β motif) which belongs to a superfamily of peptides. This motif was discovered by Bontems *et al.* (1991) and is encoded within six cysteine residues. However the motif was titled by Cornet *et al.* (1995) when the 3D structure of defensins from insect was determined.

The tertiary structure of this motif is made up of 1 α -helix linked to β -sheet with 2 strands which is kept stable by three disulfide bridges. One bridge links first β -strand with N-terminal since C1 is connected to C4. C2 – C5 maintains second bridge while C3 – C6 maintains

the third bridge thereby joining the α -helix to the C-terminal of β -strand, to form the motif (Tamaoki *et al.*, 1998).

The structure of defensins has been suggested to be part of the reason that enable them to unsettle cell membranes of organisms. For example, De Samblanx *et al.* (1997) did a structure-activity analysis of a radish defensin (Rs-AFP2) which interfere with growth of fungal hyphae in order to find the important residues that inhibit growth of fungi. Nine analogues of Rs-AFP2 were produced in which some amino acid residues were changed to that of non-antifungal plant defensin Sla2.

Real-Time RT-qPCR was done in order to profile the expression pattern of the defensin peptide, *VsDef1* in *Vigna subterranea*. The quality of RNA is the essential part of generating important data with Real-Time RT-qPCR because purified RNA is highly unstable and prone to spontaneous degradation when in solution due to its structure.

Therefore, it is crucial to treat RNA with RNase-free DNase and proceed with PCR or store at -70°C until further use. An RT-qPCR starts by selectively converting only RNA corresponding to protein-encoding genes into cDNA with aid of gene-specific primers. The reaction is catalysed by reverse transcriptase, which is an enzyme lacking RNase H activity that produces cDNA by extending oligodeoxynuceotides crossed with corresponding mRNA. The enzyme is deactivated and the duplex of RNA/DNA is broken right before the amplification starts.

In terms of reference gene validation (Figure 18), β -tubulin is therefore not a good reference gene for *V. subterranea* during the early stages of germination. Future studies will screen other candidates such as alpha tubulin to identify a gene whose express level is constant during germination.

However, the results are consistent with the expectation that high amounts of *VsDef1* (Figure19) are required in the early stages of germination in the non-sterile soil. As the plant emerges from the soil, the infection pressure decreases since the greater part of the plant is above-ground, and exposed to fewer pathogens.

CHAPTER FIVE

5.1. Conclusion

One novel defensin gene, *VsDef1* was isolated from *V. subterranea* and phylogenetically characterized; it was found to contain an intron at the genomic level.

In conclusion, *VsDef1* belongs to defensin family based on the molecular and structural analysis.

This study has yielded important resources that will provide genetic constructs which will be helpful for future research. This will enable appropriate utilization of the novel peptides, in order to answer essential questions i.e. mode of action of plant defensin and its regulation while interacting with pathogens.

5.2. Recommendation

It is essential to determine more antimicrobial peptides from *V. subterranea* and the expression pattern. Analysis of gene expression is becoming essential in various biological researches. Since patterns of expressed genes will enable understanding of complex regulatory networks, this will likely enable identifying of genes involved in diseases and new biological processes. Further studies are required to determine how the defensin responds when pathogens infect *Vigna subterranea*.

CHAPTER SIX

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APPENDICES

A1.1.

Query	1	ATGGACAAGAAATCACTAGG	TGGCTGTGCTTCCTCTTCTCCTCGTTCTTTTGTTCAGAA	60	VsDef P2 F1		
<i>Cicer arietinum</i>	1	60			
<i>Cicer arietinum</i>	77	136			
<i>Cicer arietinum</i>	879	885			
<i>Cicer arietinum</i>	747	803			
<i>Medicago truncatula</i>	41G.....A.....A.....A.....C.....	100			
<i>Medicago sativa</i>	1G.....A.....A.....A.....C.....	60			
<i>Cajanus cajan</i>	99G.....A.G.....A.....TC.....	158			
<i>Vicia faba</i>	1G.....G.....C.....C.....A.....	60			
<i>Trigonella foenum-graecum</i>	1G.....G.....G.....G.....C.....	60			
Synthetic construct defensin	1G.....G.....G.....G.....C.....	60			
<i>Psophocarpus tetragonolobus</i>	1G.....G.....G.....G.....GC.....TC.....	60			
<i>Pisum sativum</i>	78G.....G.....G.....G.....T.....C.....C.....	137			
<i>Vicia faba</i>	1G.....G.....G.....G.....C.....A.....	60			
<i>Vicia faba</i>	1G.....G.....G.....G.....C.....	60			
<i>Arachis diogoi</i>	1G.....G.....G.....G.....C.....	60			
<i>Pisum sativum</i>	56G.....G.....G.....G.....T.....C.....C.....A.....	115			
<i>Vicia faba</i>	1G.....G.....G.....G.....A.....C.....	60			
<i>Tephrosia villosa</i>	1G.....G.....G.....G.....A.....TC.....	60			
<i>Medicago truncatula</i>	1418G.....G.....G.....G.....A.....	1424			
<i>Medicago truncatula</i>	153G.....G.....G.....G.....	159			
<i>Medicago truncatula</i>	1G.....G.....G.....G.....A.....A.....	57			
<i>Trigonella foenum-graecum</i>	1G.....G.....G.....G.....	57			
<i>Medicago sativa</i>	1G.....G.....G.....G.....A.....	57			
<i>Medicago truncatula</i>	1G.....G.....G.....G.....A.....A.....	57			
<i>Datura stramonium</i>	241G.....G.....G.....G.....	276			
Query	61	GAAATAGCGGTG---AG--TGAAGCA	GCGAGGTGTGAGAAAT	TGGCTGATACATACAGGG		115	VsDef P1
<i>Cicer arietinum</i>	61		115	
<i>Cicer arietinum</i>	137		191	
<i>Cicer arietinum</i>	886		940	
<i>Medicago truncatula</i>	101T.T.....C.....TAA..CT.....A.....	155			
<i>Medicago sativa</i>	61T.T.....C.....TAA..CT.....A.....	115			
<i>Cajanus cajan</i>	159G.G.T.CAA---C---G..AA..C..C.....C.....	213			
<i>Vicia faba</i>	61T.....C---G..AGA.CT.....C.....	115			
<i>Trigonella foenum-graecum</i>	61T.T.....C---CAA..CT.....A.....	115			
Synthetic construct defensin	61T.T.....C---CAA..CT.....A.....	115			
<i>Psophocarpus tetragonolobus</i>	61G.T.T..TC---AAC.....AA..CC..C.....CC.....G.T.....	118			
<i>Pisum sativum</i>	138T.....AAC.CA.....G.T.....T.A.....	192			
<i>Vicia faba</i>	61T.....C---G..AGA.CT.....C.....	115			
<i>Vicia faba</i>	61T.....C---G..AGA.CT.....C.....	115			
<i>Arachis diogoi</i>	61T.T.....C---CAA..CT.....A.....	115			
<i>Pisum sativum</i>	116T.....AAC.CA.....G.T.....T.A.....	170			
<i>Vicia faba</i>	61T.....G..AA..CT.....C..T.A..C..T.A.....	115			
<i>Tephrosia villosa</i>	61G.T.T..CAA---G..AA..CA..C.....CC.....A..G..T.....	118			
<i>Medicago truncatula</i>	1425T.T.....C---AAC.CT.....G.....T.....	1479			
<i>Medicago truncatula</i>	160T.T.....C---TAA..CT.....A.....	214			
Query	116	GACCATGT-TT-CACAAC	TGGTAGCTGTGATGATCACTGTAAGAA-CA-AAGAGCATTTA	171	VsDef P2 R1		
<i>Cicer arietinum</i>	116	171			
<i>Cicer arietinum</i>	192	247			
<i>Cicer arietinum</i>	941	996			
<i>Medicago truncatula</i>	156C---T.G---AC.....C.CT.C.....A..GC..205				
<i>Medicago sativa</i>	116C---T.G---AC.....C.CT.C.....A..GC..165				
<i>Cajanus cajan</i>	214T.....C.....C.....T.....C.....G.....CC.G..269				
<i>Vicia faba</i>	116C-C.....G.A..C.....C.....G.....171				
<i>Trigonella foenum-graecum</i>	116C---G---CAC..T..C.CA.C---A..CGC..165					
Synthetic construct defensin	116C---G---CAC..T..C.CA.C---A..CGC..165					
<i>Psophocarpus tetragonolobus</i>	119T.....G.C.....CC.A..C.....C.....GGCGC..242				
<i>Pisum sativum</i>	193GT.....C---GGA..C.....C.....C.....171				
<i>Vicia faba</i>	116C-C.....GGA..C.....C.....C.....171				
<i>Vicia faba</i>	116C-C.....GGA..C.....C.....C.....171				
<i>Arachis diogoi</i>	116GT.....C---GGA..C.....C.....C.....GGCGC..220				
<i>Pisum sativum</i>	171GT.....C---GGA..C.....C.....C.....GGCGC..220				
<i>Vicia faba</i>	116GT.....C---GGA..C.....C.....C.....GGCGC..220				
<i>Vicia faba</i>	119T.....C---GGA..C.....C.....T.....G.....C.....174				
<i>Tephrosia villosa</i>	119T.....C---GGA..C.....C.....T.....G.....C.....174				
<i>Medicago truncatula</i>	1480T.....GTT.....CA..A.....CTT.....C.....1535				
<i>Medicago truncatula</i>	215C---T---G.....AC.....C.CT.C---A..GC..264					
Query	172	GTTAGCGGCAGATGCAGGGATGACTTTCGTTGTT	GGTGCACCAAAAATTTGTTAA	225		VsDef P2 R1	
<i>Cicer arietinum</i>	172	225			
<i>Cicer arietinum</i>	248	288			
<i>Cicer arietinum</i>	997	1050			
<i>Medicago truncatula</i>	206G.....T..T.....C.....259				
<i>Medicago sativa</i>	166G.....T..T.....C.....GA.....219				
<i>Cajanus cajan</i>	270	A.C..T.....G.....C..T.....C.....G.....C.....323				
<i>Vicia faba</i>	172	A.C..T.....181181				
<i>Trigonella foenum-graecum</i>	166G.....C..C.....T..T.....GA.....219				
Synthetic construct defensin	166G.....C..C.....T..T.....GA.....211				
<i>Psophocarpus tetragonolobus</i>	175	C.CC.T.....G.....C..T.AC.....C.....228				
<i>Pisum sativum</i>	243	A.C..T.....G.....C..T.....C.....T.....C.....296				
<i>Vicia faba</i>	172	A.C..T.....181181				
<i>Vicia faba</i>	172	A.C..T.....181181				
<i>Arachis diogoi</i>	166G.....C..C.....C..C.....T..T.....GA.....219				
<i>Pisum sativum</i>	221	A.....T..A..G.....T.....C.....274				
<i>Vicia faba</i>	172	C..T..A..G.....T.....C.....T.G.....C.....225				
<i>Vicia faba</i>	175	C.G..T..A..G.....C..T.....T..T.....GA.....228				
<i>Tephrosia villosa</i>	1536T.....G.....C.....T.G.....C.....1589				
<i>Medicago truncatula</i>	265G.....T.G.....C.....318				

Figure 20: Blast Result of Antimicrobial peptides nucleotide sequences from leguminous plants for VsDef P1 and VsDef P2 primers design

A1.2.

Query	1	ATGGAGAAGAAA	TCACTAGCTGGCTTGTGCT	CCCTC-T--TCCTCGTTCCTTTTGTGCA	57
<i>Arachis diogoi</i>	1				57
<i>Trigonella foenum-graecum</i>	1				57
Synthetic construct defensin	1				57
<i>Medicago sativa</i>	75				131
<i>Medicago truncatula</i>	43				99
<i>Trigonella foenum-graecum</i>	536				539
<i>Trigonella foenum-graecum</i>	153				156
<i>Medicago truncatula</i>	1				57
<i>Pisum sativum</i>	78				134
<i>Medicago truncatula</i>	153				156
<i>Medicago truncatula</i>	1				57
<i>Medicago truncatula</i>	59				115
<i>Cicer arietinum</i>	77				133
<i>Cicer arietinum</i>	1				57
<i>Tephrosia villosa</i>	1				57
<i>Vicia faba</i>	1				57
<i>Pisum sativum</i>	69				128
<i>Cajanus cajan</i>	99				155
<i>Psophocarpus tetragonolobus</i>	1				57
<i>Pisum sativum</i>	3				36
<i>Medicago sativa</i>	58				97
<i>Cicer arietinum</i>	747				803
<i>Medicago truncatula</i>	3614				3668
<i>Datura stramonium</i>	241				276
Query	58	CAAGAAATTGTGGTG	---A--CTGAAGCCAAAACCTTGTGAGAATTGGCTGATAAATACA		112
<i>Arachis diogoi</i>	58				112
<i>Trigonella foenum-graecum</i>	58				112
Synthetic construct defensin	58				112
<i>Medicago sativa</i>	132				186
<i>Medicago truncatula</i>	100				154
<i>Trigonella foenum-graecum</i>	540				538
<i>Trigonella foenum-graecum</i>	58				58
<i>Medicago truncatula</i>	157				211
<i>Medicago truncatula</i>	58				58
<i>Pisum sativum</i>	135				189
<i>Medicago truncatula</i>	157				211
<i>Medicago truncatula</i>	58				58
<i>Medicago truncatula</i>	116				170
<i>Cicer arietinum</i>	134				188
<i>Cicer arietinum</i>	58				112
<i>Tephrosia villosa</i>	58				115
<i>Vicia faba</i>	58				112
<i>Vicia faba</i>	58				112
<i>Pisum sativum</i>	129				183
<i>Cajanus cajan</i>	156				210
<i>Psophocarpus tetragonolobus</i>	58				115
<i>Pisum sativum</i>	37				91
<i>Medicago sativa</i>	58				58
<i>Cicer arietinum</i>	804				804
<i>Medicago truncatula</i>	3669				3669
Query	113	GGGACCATGCG-TTC	---A---G---T-G---G---C---TG---G--ACACTCATGTCACA		150
<i>Arachis diogoi</i>	113				150
<i>Trigonella foenum-graecum</i>	113				150
Synthetic construct defensin	113				150
<i>Medicago sativa</i>	187				224
<i>Medicago truncatula</i>	155				192
<i>Trigonella foenum-graecum</i>	595				632
<i>Medicago truncatula</i>	212				249
<i>Pisum sativum</i>	190				227
<i>Medicago truncatula</i>	212				249
<i>Medicago truncatula</i>	171				185
<i>Cicer arietinum</i>	189				232
<i>Cicer arietinum</i>	113				156
<i>Tephrosia villosa</i>	116				159
<i>Vicia faba</i>	113				156
<i>Vicia faba</i>	113				127
<i>Pisum sativum</i>	184				198
<i>Cajanus cajan</i>	211				254
<i>Psophocarpus tetragonolobus</i>	116				159
<i>Pisum sativum</i>	92				106
Query	151	ACCAAAGAACACGCAGTT	AGCGGCAGGTGACGGGACGACTTC	CCGCTGCTGGTGTACTAA	209
<i>Arachis diogoi</i>	151				209
<i>Trigonella foenum-graecum</i>	151				209
Synthetic construct defensin	151				209
<i>Medicago sativa</i>	151				209
<i>Medicago sativa</i>	225				283
<i>Medicago truncatula</i>	193				251
<i>Trigonella foenum-graecum</i>	633				691
<i>Medicago truncatula</i>	250				308
<i>Pisum sativum</i>	228	..AC...GGG...A..A.....T.....T.....T.....C.....		286
<i>Medicago truncatula</i>	250GA.T.....T.....T.....T.....T.....C...G		308
<i>Cicer arietinum</i>	233	.A.....G...TTT.....A.....T.....T.....T.....C...		289
<i>Cicer arietinum</i>	157	.A.....G...TTT.....A.....T.....T.....T.....C...C..		215
<i>Tephrosia villosa</i>	160	.AT..G...G...TT.C.G-	..T.A.....T.....T.....T.....C...G		218
<i>Vicia faba</i>	157	.A...T..G...TT.C.-	..T.A.....T.....T.....T.....C...G		215
<i>Cajanus cajan</i>	255	.AT.....T.A.C.T.....T.....T.....T.....T.....C.C.G		313
<i>Psophocarpus tetragonolobus</i>	160	.A.....G...TT.C.C-C.T.T.A.....T.....T.....C...C..		218
Query	210	AAGATGTAA		219	219
<i>Arachis diogoi</i>	210			219	219
<i>Trigonella foenum-graecum</i>	210			219	219
Synthetic construct defensin	210			216	216
<i>Medicago sativa</i>	210			219	219
<i>Medicago sativa</i>	284			293	293
<i>Medicago truncatula</i>	252	..AC.....		261	261
<i>Trigonella foenum-graecum</i>	692			701	701
<i>Medicago truncatula</i>	309			318	318
<i>Pisum sativum</i>	287	..AC.....		296	296
<i>Medicago truncatula</i>	309	..AC.....		318	318
<i>Cicer arietinum</i>	216	..AT.....		225	225
<i>Tephrosia villosa</i>	219			228	228
<i>Vicia faba</i>	216	..AC.....		225	225
<i>Cajanus cajan</i>	314	..AC.....		323	323
<i>Psophocarpus tetragonolobus</i>	219	..AT.....		228	228

VsDef P3 F1

VsDef P3 R1

Figure 21: Blast Result of Antimicrobial peptides nucleotide sequences from leguminous plants for VsDef P3 primer design

A1.3.

Query	1	ATGGGTCGCTCTGAGC	CTTTGGTCTCAACCATCTTTGTGTTTCTTCTGCTACTGGTGGCC	60	VsDef P5 F1	
<i>Vigna unguiculata</i>	1	60		
<i>Vigna angularis</i>	42364335C..C.....T.....	42364394		
<i>Vigna angularis</i>	74C..C.....T.....	133		
<i>Vigna radiata</i>	86C.....T.....	145	VsDef P4 F1	
Query	61	ACTGGTAAGCCTTTCTCCACGC	TCAATACCATCAttttagggttttcttttaacattttc	120		
<i>Vigna unguiculata</i>	61	120		
<i>Vigna angularis</i>	42364395C..T.....	42364452		
<i>Vigna angularis</i>	134	135		
<i>Vigna radiata</i>	146	147		
Query	121	tttttctctttttGATTGTCAATAAGATTATTCAAGTTGTATGTATATATATATGAA	180	180	VsDef P5 R1	
<i>Vigna unguiculata</i>	121	180		
<i>Vigna angularis</i>	42364453C.....	42364469	180		
Query	181	CTAGATCGTGTTAATTACGTTGTGTTTTTGAATGAGTTGAGCAGA-GATGGGGCCAA---	236	236	VsDef P4 R1	
<i>Vigna unguiculata</i>	181	236		
<i>Vigna angularis</i>	42364462T..T.....	42364490		
<i>Vigna angularis</i>	137	149		
<i>Phaseolus vulgaris</i>	114A.....	126		
<i>Phaseolus vulgaris</i>	299A.....	311		
<i>Vigna radiata</i>	149	161		
<i>Cajanus cajan</i>	135	147		
<i>Glycine max</i>	117T.....	131		
<i>Glycine max</i>	113T.....	127		
<i>Glycine max</i>	118T.....	132		
<i>Glycine max</i>	111T.....	125		
<i>Phaseolus vulgaris</i>	86A.....	98		
<i>Nicotiana benthamiana</i>	62A.....	76		
<i>Nicotiana alata</i>	62A.....TGA	79		
Query	237	CAATGGTGGCAGAGGCAAGGACCTGTGAGTCTCAGAGCCACCGTTTCAAGGGACCATGTG	296	296		
<i>Vigna unguiculata</i>	237	296		
<i>Vigna angularis</i>	42364491G.....A.....C.	42364550		
<i>Vigna angularis</i>	150G.....A.....C.	209		
<i>Phaseolus vulgaris</i>	127	G.....	G.....A..T.....	186		
<i>Phaseolus vulgaris</i>	312	G.....	G.....A..T.....	371		
<i>Vigna radiata</i>	162G.....A..C.....	221		
<i>Cajanus cajan</i>	148A.....A.....A.....	207		
<i>Glycine max</i>	132A.....A..T.....G.....T	191		
<i>Glycine max</i>	128A.....A..T.....G.....T	187		
<i>Glycine max</i>	133A.....A..T.....G.....T	192		
<i>Glycine max</i>	126A.....A..T.....G.....T	185		
<i>Phaseolus vulgaris</i>	99	G.....	G.....AGGT.....G.....G..A..C.....G.....AA.....	158		
<i>Nicotiana benthamiana</i>	77	.G.CAA.T.....	A.....	134		
<i>Nicotiana alata</i>	80A..T..C.....	136		
Query	297	TGAGTGACACCAACTGTGCTTCTGTTTGCCGAAGTGAACGTTTCAGCG	GAGGACATTGCC	356	VsDef P4 R1	
<i>Vigna unguiculata</i>	297	356		
<i>Vigna angularis</i>	42364551C.....	42364610		
<i>Vigna angularis</i>	210C.....	269		
<i>Phaseolus vulgaris</i>	187C.....	246		
<i>Phaseolus vulgaris</i>	372C.....	431		
<i>Vigna radiata</i>	222A.....C.....	281		
<i>Cajanus cajan</i>	208G.....C.....T.....G.....CT.....	267		
<i>Glycine max</i>	192GC.....C.....CT.....	251		
<i>Glycine max</i>	188GC.....C.....CT.....	247		
<i>Glycine max</i>	193GC.....C.....CT.....	252		
<i>Glycine max</i>	186GC.....C.....CT.....	245		
<i>Phaseolus vulgaris</i>	159	A.....	GAG.....CC.....	218		
<i>Nicotiana alata</i>	137	CA..A..T.G.....	155		
Query	357	GTGGCTTCCGTCGCAGATGCTTGTCCACCAAACTGTTA	397	397		VsDef P5 R1
<i>Vigna unguiculata</i>	357	397		
<i>Vigna angularis</i>	42364611C.....T.....T.....	42364651		
<i>Vigna angularis</i>	270C.....T.....T.....	310		
<i>Phaseolus vulgaris</i>	247T.....C.....	287		
<i>Phaseolus vulgaris</i>	432T.....C.....	472		
<i>Vigna radiata</i>	282C.....C.....T.....	320		
<i>Cajanus cajan</i>	268C.....	308		
<i>Glycine max</i>	252C.....T.....	292		
<i>Glycine max</i>	248C.....T.....	288		
<i>Glycine max</i>	253C.....T.....	293		
<i>Glycine max</i>	246C.....T.....	286		
<i>Phaseolus vulgaris</i>	219T.T.....C.....GA.....G..C.....	258		

Figure 22: Blast Result of Antimicrobial peptides nucleotide sequences from leguminous plants for VsDef P4 and VsDef P5 primers design

A2.1.

Cleaning of consensus sequences of VsDef P5 F1 and VsDef P5 R1 PCR products

	5	15	25	35	45	55
VsDef F1	TTTAACAGTG	TTTGGTGCAC	AAGCATCTGC	GACG-GAAGC	CACG-GCAAT	GTCCTCCGCT
VsDef R1	-----	-----	-----	---GCGAAG-	-ACGTATAAT	GTACTCCGTA
Consensus	TTTAACAGTG	TTTGGTGCAC	AAGCATCTGC	GACGCGAAGC	CACGTACAAT	GTCCTCCGCT

	65	75	85	95	105	115
VsDef F1	GAAACGTTG-	GGTCCGGCAA	ACGGAAGCAC	AGTTGGTGTT	ACTCACACAT	GGTCCCTTGA
VsDef R1	CATACGTTCA	CGT-CGAACA	ACGG-AGCAC	AAATGTCGTT	ACTCATTCAT	GGAACCTTG-
Consensus	GAAACGTTCA	GGTCCGGCCA	ACGGAAGCAC	AGATGTCGTT	ACTCACACAT	GGTCCCTTGA

	125	135	145	155	165	175
VsDef F1	AACGGTGGCT	CTGAGACTCA	CAGGTCCTTG	CCTCTGCCAC	CATTGTTGGC	CCCATCTCTG
VsDef R1	AACGGAGCCT	CTGAGACTCA	CAGGTCCTTG	CCTCTGCCAC	CATTGTTGGC	CCCATCTCTG
Consensus	AACGGTGCCT	CTGAGACTCA	CAGGTCCTTG	CCTCTGCCAC	CATTGTTGGC	CCCATCTCTG

	185	195	205	215	225	235
VsDef F1	CTCAACTCAT	TCAAAAACAC	AACTTAATTA	ACACGCTCTG	GTTCGTAAAT	ATGCACAAAC
VsDef R1	CTCAACTCAT	TCAAAAACAC	AACTTAATTA	ACACACTCTG	GTTCGTAAAT	ATGCACAAAC
Consensus	CTCAACTCAT	TCAAAAACAC	AACTTAATTA	ACACGCTCTG	GTTCGTAAAT	ATGCACAAAC

	245	255	265	275	285	295
VsDef F1	TTCAATAATC	TTGTTACAAA	TAAATGAGAA	AAAAAATGT	TGCAATAG-A	GACCCTAAGA
VsDef R1	TTCAATAATC	TTGTTACAAA	TAAATGAGAA	AAAAAATGT	TGAAATAGAA	AACCCTAAGA
Consensus	TTCAATAATC	TTGTTACAAA	TAAATGAGAA	AAAAAATGT	TGAAATAGAA	AACCCTAAGA

	305	315	325	335	345	355
VsDef F1	TGATAGT-AT	GAGCGCGAAG	AAAGGCAAAC	CAGT-GTCAC	CAGACATAGA	CTTAA-----
VsDef R1	TGATAGTGTT	GAGCGCGAAG	AAAGGCTTAC	CAGTGGCCAC	CAGAAGCAGA	AGAAACACAA
Consensus	TGATAGTGTT	GAGCGCGAAG	AAAGGCTAAC	CAGTGGCCAC	CAGAAGCAGA	AGAAACACAA

	365	375	385	395	405	415
VsDef F1	-----	-----	-----	-----	-----	-----
VsDef R1	AGATGGTTGA	GACCAAAGGC	ACAGAGCGAG	CCAAGGGGAG	GAAAGAAAAG	GCTTGTGCAC
Consensus	AGATGGTTGA	GACCAAAGGC	ACAGAGCGAG	CCAAGGGGAG	GAAAGAAAAG	GCTTGTGCAC
				
	425	435				
VsDef F1	-----	-----				
VsDef R1	CAAACACTGT	TAAAAA				
Consensus	CAAACACTGT	TAAAAA				

A2.4.

Alignments of *VsDef1* and *Vigna unguiculata* defensin precursor (PDEF) gene.

Score	Expect	Identities	Gaps	Strand	Frame													
512 bits(277)	8e-150()	362/401(90%)	14/401(3%)	Plus/Minus														
Query 2	TTAACAGTGT	TTTGGTGCACAAGCATCTGCGACG	C	GAAGCCACG	T	ACAATGTCTCCGCTG 61												
Sbjct 397	TTAACAGTGT	TTTGGTGCACAAGCATCTGCGACG	-	GAAGCCACG	-	GCAATGTCTCCGCTG 340												
Query 62	AAACGTT	CAGGTC	CGGCAAACG	GAAGCACAG	ATGTCGT	TA	CTCACACATGGTCCCTTGAA 121											
Sbjct 339	AAACGTT	CAG-TTCGGCAAACAGAAGCACAG	T	TGGTGTCACTCACACATGGTCCCTTGAA 281														
Query 122	ACGGTG	C	CTCTGAGACTCACAGGTCCTTGCCCTCTGCCACCATTGTTGGCCCCATCTCTGC 181															
Sbjct 280	ACGGTGGCTCTGAGACTCACAGGTCCTTGCCCTCTGCCACCATTGTTGGCCCCATCTCTGC 221																	
Query 182	TCAACTCATTCAAAAACACAAC	T	TAATTAACACG	C	TCT	G	TTC	G	TA	-	A	-	TGC	--	A 235			
Sbjct 220	TCAACTCATTCAAAAACACAACGTAATTAACACGATCTAGTTCATATATATACATACATA 161																	
Query 236	CAAAC	T	CAATAATCTT	G	T	C	ACAAT-AAA	T	-	GAG	aaaaa	-	aaaa	TGTT	G	AAA	T	AGAAAA 292
Sbjct 160	CAAAC	T	GAAATAATCTT	A	T	T	GACAATCAAAAAGAGAAAAAGAAAATGTT-AAA-AGAAAA 103											
Query 293	CCCTAAG	A	TGATAGT	C	T	T	GAGCGC	G	A	AGAAAGGCT	A	ACCAGTGGCCACCAGA	A	AGCAGAAG 352				
Sbjct 102	CCCTAAAATGATGGTATTGAGCGTGGAGAAAGGCTTACCAGTGGCCACCAGTAGCAGAAG 43																	
Query 353	AAACACAAAGATGGTTGAGACCAAAGGCACAGAGCGAGCCA 393																	
Sbjct 42	AAACACAAAGATGGTTGAGACCAAAGGCACAGAGCGAGCCA 2																	

Table 2: Justification for changes in the nucleotide sequences

Position 35 – C deleted	Position 128 – G based on VUG F1 trace
Position 45 – T deleted	Position 215 – G or A based on clear peaks on VUG F1 and R1 traces
Position 72 – G deleted	Position 216 to 225 - confirmed significant difference
Position 74 –	Position 225 to 235 - confirmed significant difference since the gaps restores reading frame
Position 83 – confirmed significant difference	Position 243 to 265 - confirmed significant difference
Position 93 – T but it's a on reverse strand (VUG R1 trace)	Position 261 to 282 - confirmed significant difference since the gaps restores reading frame
Position 96 – G or T based on clear peaks on VUG F1 and R1 traces	Position 299 – confirmed significant difference

A3.1.

Traces from Real Time PCR analysis

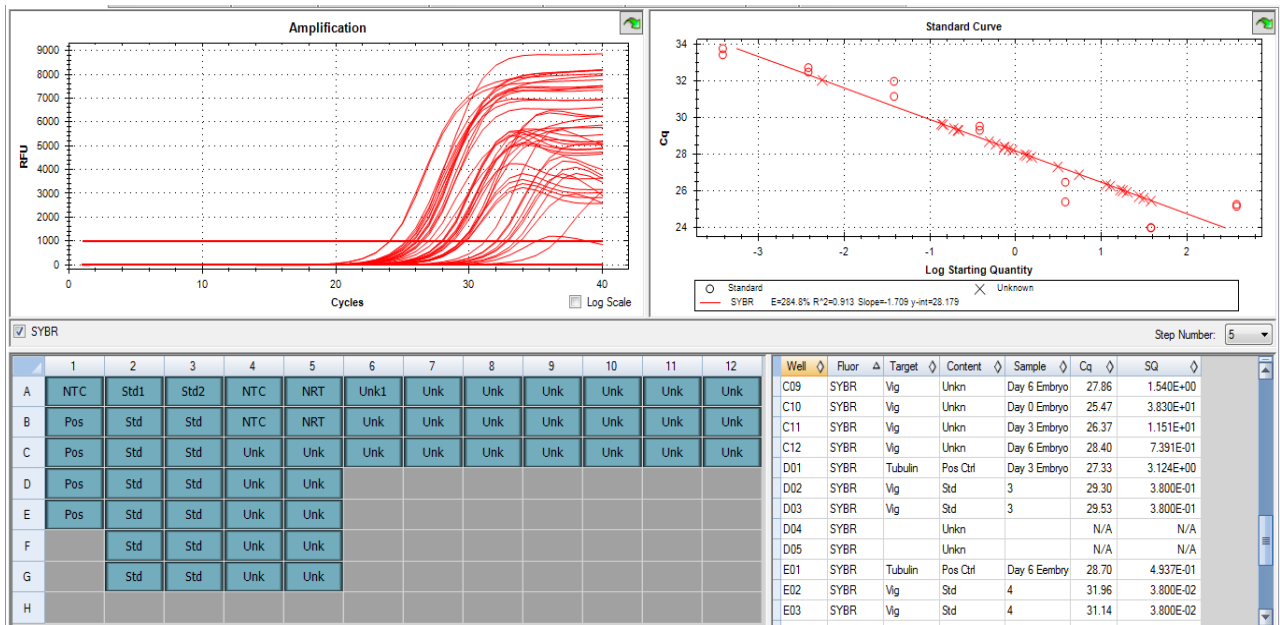


Figure 26: showing analysis of standard curve done using a Bio-Rad CFX96 Real-Time system. Panel (left) linear plot; (right) standard curve. X – axis is cycle number; Y – axis is amount of RNA. Five-fold serial dilutions of RNA samples were run so as to create melting curve (circle). The concentration of mRNA in the samples, run in technical triplicates, was measured from the plot of the C_T values (crosses). C_T = cycle threshold

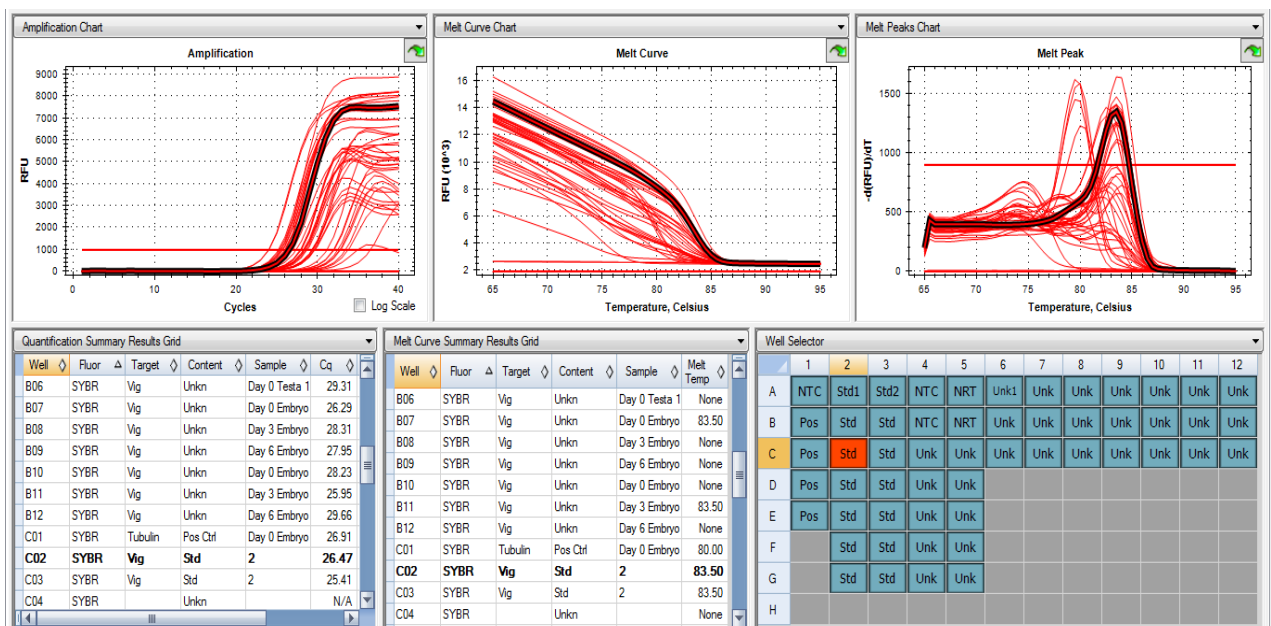


Figure 27: showing analysis of melt curve done on a Bio-Rad CFX96 real-time PCR system. Panel (right) shows 2 peaks which could indicate primer-dimer, unspecific binding of the VsDef P4 primers or misprimed products.

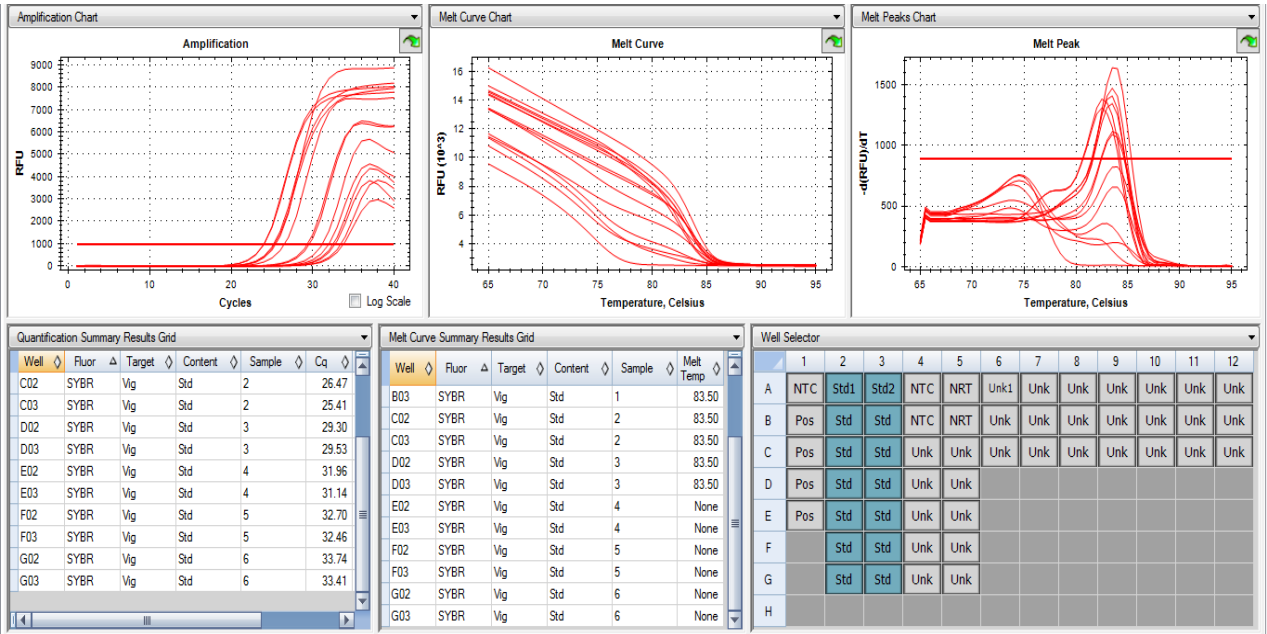


Figure 28: showing analysis of melt curve done on a Bio-Rad CFX96 real-time PCR system. Panel (right) shows several peaks which could indicate primer-dimer, unspecific binding of the VsDef P4 primers or misprimed products.

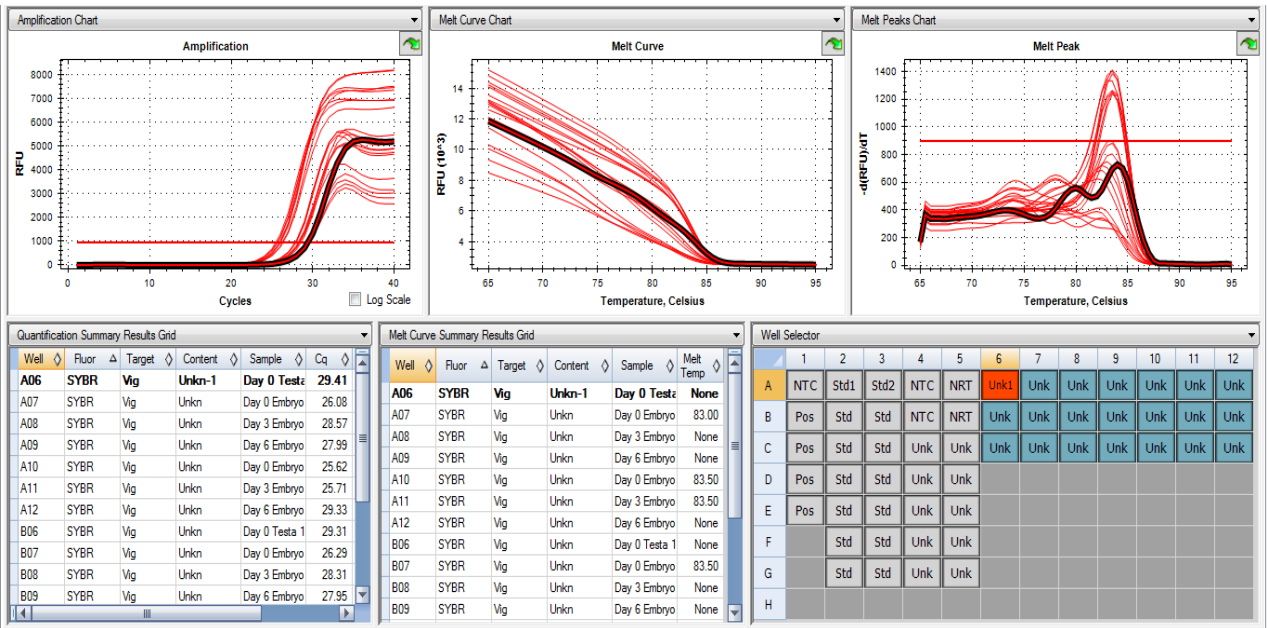


Figure 29: analysis of melt curve done on a Bio-Rad CFX96 real-time PCR system. Panel (right) shows many peaks which could indicate primer-dimer, unspecific binding of the VsDef P4 primers or misprimed products.

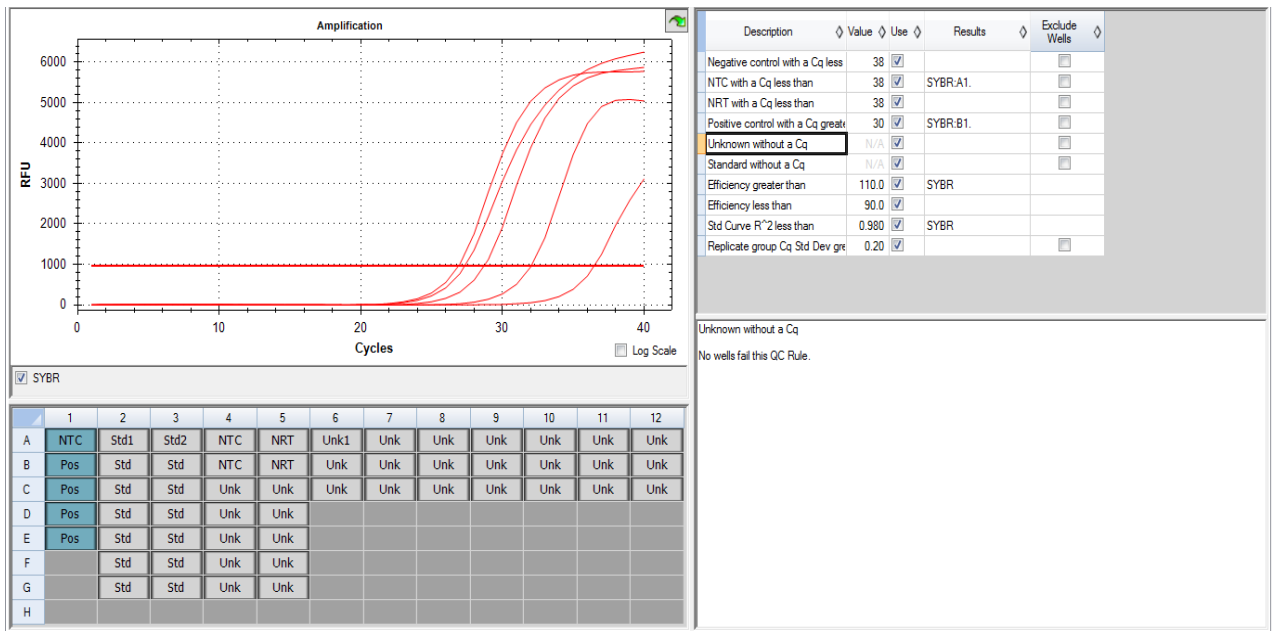


Figure 30: Photograph showing concentration of tubulin vs *V. subterranea* mRNA